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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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TITLE OF THE INVENTION (280 characters max)

CCL21, IN COMBINATION WITH BACULOVIRUS, POMOTES CANCER RESISTANCE

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ENCLOSED APPLICATION PARTS *(check all that apply)*

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Respectfully submitted,

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USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C.

P19LARGE/REV05

CCL21, in Combination with Baculovirus, Promotes Cancer Resistance

Field of the Invention

[0001] The present invention generally relates to cancer therapy. More particularly, the present invention relates to improved methods for CCL21 immunotherapy, wherein the improvement relates to co-administration of an adjuvant.

Table of Abbreviations

[0002]

A549	-	human lung epithelial tumor cell line
AcNPV	-	*Autographa californica* nucleopolyhedrosis virus
BMDC	-	bone marrow-derived dendritic cells
BV422	-	recombinant baculovirus expressing CCL21
BV762	-	recombinant baculovirus expressing Raf
CCL21	-	C-C motif Ligand 21 Chemokine; Secondary Lymphoid-Tissue Chemokine
CD86	-	marker for dendritic cell maturation
CR	-	complete response
CTL	-	cytotoxic T lysis
DC	-	dendritic cell
FACS	-	fluorescence activated cell sorting
GM-CSF	-	Granulocyte-Macrophage Colony-Stimulating Factor
GV	-	granulosis virus
HIV	-	human immunodeficiency virus
i.t.	-	intratumorally
mCCL21	-	mouse CCL21
MHC	-	major histocompatibility complex
MHC II	-	MHC class II
MLA-DR	-	MHC class I antigen

MOI	-	multiplicity of infection
NPV	-	nucleopolyhedrosis virus
PBMCs	-	peripheral blood mononuclear cells
PFU	-	plaque-forming unit
qd	-	Quaque Die (given daily)
rhCCL21	-	recombinant human CCL21
s.c.	-	subcutaneously
Sf (Sf9)	-	*Spodoptera frugiperda*
Tn (Tn5)	-	*Trichoplusia ni*
UV	-	ultraviolet
VLP	-	virus-like particle

Description of Related Art

[0003] Modulation of immune response has become an important anti-cancer strategy. A significant effort in the design of cancer vaccines and immunotherapies has focused on the identification of antigens that are selectively present in tumor cells. Unique tumor immunogenicity has permitted induction of tumor-specific immune responses using vaccines that include tumor-specific antigens, or genes expressing tumor-specific antigens. Vaccination approaches have also included adoptive cellular methods, whereby antigen-presenting cells are modified to present tumor-associated antigens. Additional immunological strategies for cancer treatment include administration of cytokines and chemokines, which have therapeutic potential as adjuvants or treatments in anti-cancer therapies based on their ability to expand and recruit immune effector cells. *See e.g.*, Homey et al. (2002) *Nat Rev Immunol* 2:175-84; Parmiani et al. (2002) *J Natl Cancer Inst* 94:805-18; Bronte (2001) *Curr Gene Ther* 1:53-100; and Fehniger et al. (2002) *Cytokine Growth Factor Rev* 13:169-83.

[0004] Notwithstanding the above-noted advances, the success of immunological approaches has been limited by: (1) tumor-specific antigenicity, such that therapies are limited to particular cancer types; (2) poor antigen presentation by tumor cells;

and (3) and the ability of tumor cells to produce immune inhibitory factors to thereby escape immune surveillance. Thus, there exists a long-felt and continuing need in the art for effective and broadly applicable cancer therapies. To meet this need, the present invention provides novel immunostimulatory methods for cancer treatment and prevention.

Summary of the Invention

[0005] The present invention provides methods for the treatment or prevention of cancer, including tumors and non-neoplastic proliferative disorders, in a mammalian subject via administering to the subject: (a) an effective amount of a *CCL21* nucleic acid or a CCL21 polypeptide; and (b) an effective amount of an adjuvant. Thus, the present invention also provides methods for the treatment or prevention of non-neoplastic disorders in a mammalian subject via administering to the subject: (a) an effective amount of a *CCL21* nucleic acid or a CCL21 polypeptide; and (b) an effective amount of an adjuvant. In preferred embodiments of the invention, the treatment or prevention comprises inhibition of cancer growth, including complete cancer remission, inhibition of cancer metastasis, resistance to cancer re-challenge, and/or induction of cancer cell cytolysis. In addition, the methods can further comprise eliciting an innate immune response, an adaptive immune response, or a combination thereof.

[0006] The disclosed methods are directed to cancer treatment and prevention in human subjects; however, they can be used for the treatment of any mammal in need thereof. Representative cancers and non-neoplastic disorders that can be treated or prevented include those of the lung, breast, and skin.

[0007] A *CCL21* nucleic acid used in accordance with the methods of the invention can comprise: (a) an isolated nucleic acid molecule encoding a polypeptide of SEQ ID NO:2; (b) an isolated nucleic acid molecule of SEQ ID NO:1; or (c) an isolated nucleic acid molecule that is at least 90% identical to SEQ ID NO:1.

[0008] A *CCL21* nucleic acid used in accordance with the methods of the invention can also comprise: (a) an isolated nucleic acid molecule encoding a polypeptide of SEQ ID NO:2; (b) an isolated nucleic acid molecule of SEQ ID NO:1; (c) an isolated nucleic acid molecule which hybridizes to a *CCL21* nucleic acid sequence of SEQ ID

NO:1 under wash stringency conditions represented by a wash solution having less than about 200 mM salt concentration and a wash temperature of greater than about 45°C, and which encodes a CCL21 polypeptide; and (d) an isolated nucleic acid molecule differing by at least one functionally equivalent codon from the isolated nucleic acid molecule of one of (a), (b), and (c) above in nucleic acid sequence due to the degeneracy of the genetic code, and which encodes a CCL21 polypeptide encoded by the isolated nucleic acid of one of (a), (b), and (c) above.

[0009] In a preferred embodiment of the present invention, the *CCL21* nucleic acid comprises: (a) an isolated nucleic acid molecule encoding a polypeptide of SEQ ID NO:2; or (b) an isolated nucleic acid molecule of SEQ ID NO:1.

[0010] Where a CCL21 polypeptide is used to perform the methods of the present invention, the CCL21 polypeptide can comprise: (a) a polypeptide of SEQ ID NO:2; (b) a polypeptide that is at least 90% identical to SEQ ID NO:2; (c) a polypeptide encoded by a nucleic acid molecule of SEQ ID NO:1; or (d) a polypeptide encoded by a nucleic acid molecule that is at least 90% identical to SEQ ID NO:1.

[0011] A CCL21 polypeptide can also comprise a polypeptide encoded by a nucleic acid molecule selected from the group consisting of: (a) an isolated nucleic acid molecule encoding a polypeptide of SEQ ID NO:2; (b) an isolated nucleic molecule of SEQ ID NO:1; (c) an isolated nucleic acid molecule which hybridizes to a *CCL21* nucleic acid sequence under wash stringency conditions represented by a wash solution having less than about 200 mM salt concentration and a wash temperature of greater than about 45°C, and which encodes a CCL21 polypeptide; and (d) an isolated nucleic acid molecule differing by at least one functionally equivalent codon from the isolated nucleic acid molecule of one of (a), (b), and (c) above in nucleic acid sequence due to the degeneracy of the genetic code, and which encodes a CCL21 polypeptide encoded by the isolated nucleic acid of one of (a), (b), and (c) above.

[0012] Preferably, a CCL21 polypeptide used to perform the disclosed methods comprises SEQ ID NO:2.

[0013] In a preferred embodiment of the invention, an adjuvant used to perform the disclosed therapeutic methods comprises a non-pathogenic virus, including a live virus, an inactivated virus, a viral particle, a viral occlusion body, or a viral

component. Representative viral components include, for example, peptide, proteins, nucleic acids, lipids, carbohydrates, and combinations thereof.

[0014] Preferably, a non-pathogenic virus is an insect-specific virus, and more preferably a virus of the family of Baculoviridae. For example, a non-pathogenic virus of the invention can comprise a nucleopolyhedrosis virus or a granulosis virus. A particularly preferred non-pathogenic virus is *Autographa californica* nuclepolyhedrosis virus.

[0015] For the treatment of tumors, CCL21 and/or the adjuvant are preferably administered to a mammalian subject intratumorally and/or peritumorally. Similarly, for the treatment of non-neoplastic proliferative disorders, CCL21 and/or the adjuvant are preferably administered to a mammalian subject intralesionally and/or perilesionally. A therapeutic regimen can include multiple administrations of each CCL21 and/or adjuvant. CCL21 and adjuvant can be simultaneously co-administered, for example by preparation of a single composition comprising both CCL21 and adjuvant. Alternatively, CCL21 and adjuvant can be sequentially administered.

Brief Description of the Drawings

[0016] Figure 1 is a line graph, which shows that baculovirus-expressed CCL21 inhibits tumor growth and induces complete tumor remission in a 3LL mouse model of lung cancer. As described in Example 2, CCL21 administration included 6 intratumoral injections of CCL21 at the concentrations indicated. Increasing the dose per injection increased tumor inhibition and the frequency of complete responses. One way ANOVA analysis was performed using data collected on day 24. Albumin compared with treated groups, $P<0.001$; 25 µg mCCL21 compared with 6 µg/Alb mCCL21, $P<0.01$; 25 µg mCCL21 compared with 25 µg rhCCL21, $P>0.05$. mCCL21, mouse CCL21; rhCCL21, recombinant human CCL21; B Gold, recombinant mouse CCL21 expressed from baculovirus, which was used as a reference lot ("Gold Standard"); Alb, albumin; qd, Quaque Die (given daily), CR, complete response.

[0017] Figure 2 is a line graph, which depicts inhibition of tumor growth in a 4T1 mouse model of breast cancer following administration of baculovirus-expressed

CCL21, as described in Example 3. rhCCL21, recombinant human CCL21; qd, Quaque Die (given daily).

[0018] Figure 3 is a bar graph, which depicts inhibition of spontaneous 4T1 lung metastasis following administration of baculovirus-expressed CCL21, as described in Example 6. Solid bars, animals that did not undergo surgical resection of the tumor; hatched bars, animals in which tumors were surgically resected (Surg) 1 day after the last dose of CCL21.

[0019] Figure 4 is a line graph, which shows that administration of baculovirus-expressed CCL21 imparts resistance to tumor re-challenge, as described in Example 5. Briefly, 4T1 tumors were established in Balb/c mice, and a subset of host mice were treated via intratumoral administration of baculovirus-expressed CCL21. One day after the last dose of CCL21, tumors were surgically resected. One day after tumor resection, mice were re-challenged with s.c. injection of 4T1 cells at a site contralateral to the original tumor. Naïve, mice that had not previously hosted a tumor and which did not receive CCL21 treatment; Alb + Surg + Re-chlg, mice that had previously hosted a 4T1 tumor and that received albumin treatment; hCCL21 + Surg + Re-chlg, mice that had previously hosted a 4T1 tumor and that received CCL21 treatment. In the Alb + Surg + Re-chlg group, 1 of 10 mice showed complete resistance to tumor growth. In the hCCL21 + Surg + Re-chlg group, 6 of 10 mice showed complete resistance to tumor growth.

[0020] Figures 5A-5C summarize experiments that demonstrate baculovirus-induced resistance to tumor re-challenge. In the 3LL tumor model, animals that have been successfully treated with baculovirus-derived CCL21 are resistant to re-challenge with the same tumor for prolonged periods.

[0021] Figure 5A summarizes experiments with animals that had a complete remission of tumors after treatment with baculovirus-derived mouse recombinant CCL21 (*see e.g.*, Figure 1), and were re-challenged in the opposite flank with the same tumor at 60, 70 and 80 days following the last dose of baculovirus-derived CCL21. Animals were resistant to re-challenge for at least 70 days.

[0022] Figure 5B summarizes experiments with animals that received an inoculation 30 days following completion of baculovirus CCL21 treatment and induction of complete remission ("tumor boost"), and that were re-challenged in the opposite

flank with the same tumor at 80, 120, 160 and 200 days following the last dose of baculovirus-derived CCL21. These results demonstrate that resistance to tumor re-challenge can be extended to at least 200 days by using a tumor boost.

[0023] Figure 6 is a line graph, which shows that CCL21 produced in yeast or *E.coli* does not result in tumor remission in a 3LL tumor model of lung cancer. hCCL21-B (HBPG1), recombinant human CCL21 expressed in baculovirus, lot# HBPG1; hCCL21-B ½ (HBPG1), recombinant human CCL21 expressed in baculovirus, lot# HBPG1, diluted to one-half of the concentration of hCCL21 (HBPG1); hCCL21-B conc. (HBPG1), recombinant human CCL21 expressed in baculovirus, lot# HBPG1 derived from a concentrated (10 mg/ml) solution; hCCL21-Y (HYPG4), recombinant human CCL21 expressed in yeast, lot# HYPG4; hCCL21-E (HEDS4), recombinant human CCL21 expressed in *E.coli*, lot# HEDS4; qd, Quaque Die (given daily). Inhibition of tumor growth in mice treated with albumin when compared to mice treated with hCCL21-B or with hCCL21-B new, $P < 0.01$; inhibition of tumor growth in mice treated with hCCL21-B conc. or with hCCL21-B ½, $P < 0.05$.

[0024] Figure 7 is a line graph, which shows *in vitro* chemotaxis activity of baculovirus-expressed-CCL21 preparations that are inactive *in vivo*. The chemotaxis assay can be performed essentially as described in PCT International Publication No. WO 00/38706. *In vivo* activity was assessed as described in Examples 2-6. HBDS2.p, recombinant human CCL21 derived from baculovirus, lot# HBDS2.p; MBDS2.c, recombinant mouse CCL21 derived from baculovirus, lot# MBDS2.c; HBPG1, recombinant human CCL21 derived from baculovirus, lot# HBPG1; HBPG1 + HBDS2.vp, HBPG1 mixed with an equimolar ratio of HBDS2 that had been treated with vinyl pyridine; HYPG4, recombinant human CCL21 derived from yeast, lot# HYPG4; HEDS4, recombinant human CCL21 derived from E.coli, lot# HEDS4.

[0025] Figure 8 is a photograph of a Western blot that was prepared using the indicated samples and then probed with an anti-gp64 antibody. Lane 1, purified baculovirus; Lane 2, conditioned media from uninfected Tn5 cell culture; Lane 3, conditioned media from Tn5 cells infected with wild type baculovirus; Lane 4, conditioned media from Tn5 cell culture infected with BV422 encoding recombinant human CCL21; Lane 5, uninfected Tn5 cell pellet; Lane 6, cell pellet from Tn5 culture infected with wild type baculovirus; Lane 7, cell pellet from Tn5 culture infected with

BV422; Lane 8, human recombinant CCL21 derived from baculovirus, lot# HBPG1, filtrate after removing contaminants >50 kDa; Lane 9, retentate from sample in Lane 8 containing contaminants >50 kDa (5 µg of protein); Lane 10, retentate from sample in Lane 8 containing contaminants >50 kDa (10 µg of protein); Lane 11, 5 µg of unfiltered recombinant human baculovirus-derived CCL21, lot# HBPG1; Lane 12, 5 µg of unfiltered recombinant human baculovirus-derived CCL21, lot# HBDS4; Lane 13, 5 µg of unfiltered recombinant human baculovirus-derived CCL21, lot# HBMC1; Lane 14, 5 µg of unfiltered recombinant human baculovirus-derived CCL21, lot# HBDS1.

[0026] Figure 9 is a line graph, which shows that the anti-tumor activity of baculovirus-expressed CCL21 is removed by filtering to remove high molecular weight contaminants from the preparation.

[0027] Figure 10A is a bar graph, which shows changes in dendritic cell expression of CD86 and MHC II in response to the indicated stimuli. Mouse bone marrow-derived dendritic cells were prepared and analyzed as described in Example 8. Vaccinia virus expressing HIV gag protein (VLP) was used as a positive control. Similar to VLP, live baculovirus induced CD86 and MHC II expression, which is indicative of DC maturation. Black bars, CD86 expression; gray bars, MHC II expression.

[0028] Figure 10B is a bar graph, which shows changes in dendritic cell expression of CD86 and MHC II in response to the indicated stimuli. Human monocyte-derived dendritic cells were prepared and analyzed as described in Example 8. Vaccinia virus expressing the HIV gag protein (p55 VLP) was used as a positive control. Similar to p55 VLP, live baculovirus induced DC maturation. Black bars, CD86 expression; gray bars, HLA-DR expression.

[0029] Figure 11 is a plot of the results of a chromium release assay, which was performed as described in Example 9. Vaccinia virus expressing HIV gag protein (VLP) was used as a positive control. Similar to VLP, live baculovirus acts as a potent adjuvant to induce cytotoxic T cell lysis.

Brief Description of Sequences in the Sequence Listing

[0030] SEQ ID NOs:1-2 are human CCL21 nucleotide and amino acid sequences,

respectively.

Detailed Description of the Invention

[0031] The present invention provides improved anti-cancer therapies, wherein the improvement comprises co-administration of a CCL21 nucleic acid or polypeptide with an adjuvant. The disclosed methods can elicit a cancer-specific adaptive immune response in a mammalian subject. Thus, the disclosed methods are useful for inhibition of tumor growth and for conferring resistance to tumor re-challenge.

A. CCL21

[0032] Chemokines constitute a group of over 30 small (8-12 kDa), heparin-binding cytokines with common structural features that mediate leukocyte migration. In addition, some chemokines have been shown to modulate hematopoiesis, angiogenesis, suppression of apoptosis, and HIV-1 absorption. Most chemokines contain four conserved cysteine residues and are grouped into four major subfamilies, CXC, CC, C and CX_3C, based on the position of their conserved cysteine residues. In CC chemokines, the two amino terminal cysteine residues are contiguous. CC chemokines are generally chemotactic to monocytes, eosinophils, basophils, and/or lymphocytes with variable selectivity. *See* Baggiolini et al. (1997) *Annu Rev Immunol* 15:675-705; Jung & Littman (1999) *Curr Opin Immunol* 11:319-25; Homey et al. (2002) *Nat Rev Immunol* 2:175-84; and references cited therein.

[0033] Secondary Lymphoid Chemokine (SLC; also known as 6Ckine, Exodus-2, and thymus-derived chemotactic agent 4 (TCA4)), referred to herein as "CCL21," differs from most other CC chemokines in structure, chromosomal localization, pattern of tissue expression, and receptor usage. In contrast to other CC chemokines, CCL21 has been reported to be chemotactic to lymphocytes, with preferential activity toward naïve T cells, but not to monocytes or neutrophils. *See* Nagira et al. (1997) *J Biol Chem* 272: 19518-24 and Gunn et al. (1998) *Proc Natl Acad Sci USA* 95: 258-63. CCL21 also mediates chemotaxis of dendritic cells. *See* PCT International Publication No. WO 00/38706. In addition to its chemotactic functions, CCL21 has angiostatic properties. *See* Soto et al. (1998) *Proc Natl Acad Sci USA* 95:8205-10.

[0034] Recently, several groups have reported that CCL21 can inhibit the progression of cancer and other proliferative disorders. *See* Sharma et al. (2000) *J Immunol* 164:4558-63; Kirk et al. (2001) *Cancer Res* 61:2062-70; Arenberg et al. (2001) *Cancer Immunol Immunother* 49:587-92; PCT International Publication No. WO 00/38706; and U.S. Patent No. 6,096,300.

[0035] The present invention relates to and extends the findings of the above-noted studies by demonstrating that adjuvant is required for CCL21 anti-cancer activity. Based on this discovery, the present invention further provides improved CCL21 therapies, which comprise co-administration of CCL21 and an adjuvant.

[0036] The term "CCL21" and terms including "CCL21" (*e.g.*, hCCL21) refer generally to isolated *CCL21* nucleic acids, isolated polypeptides encoded by *CCL21* nucleic acids, and activities thereof. CCL21 nucleic acids and polypeptides can be derived from any organism. Preferably, the CCL21 nucleic acids and polypeptides are derived from the same organism in need of treatment. A representative *CCL21* nucleic acid and CCL21 polypeptide, which can be used in accordance with the disclosed methods, are set forth as SEQ ID NOs:1 and 2, respectively.

[0037] The term "isolated", as used in the context of a nucleic acid or polypeptide, indicates that the nucleic acid or polypeptide exists apart from its native environment and is not a product of nature. An isolated nucleic acid or polypeptide can exist in a purified form or can exist in a non-native environment such as a transgenic host cell.

[0038] As disclosed further herein below, the present invention also provides a system for functional expression of a CCL21 polypeptide. The system employs a recombinant CCL21 nucleic acid, including SEQ ID NO:1.

A.1 *CCL21* Nucleic Acids

[0039] The terms "nucleic acid molecule" and "nucleic acid" each refer to deoxyribonucleotides or ribonucleotides and polymers thereof in single-stranded, double-stranded, or triplexed form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar properties as the reference natural nucleic acid. The terms "nucleic acid molecule" or "nucleic acid" can also be used in place of "gene," "cDNA," "mRNA," or "cRNA." Nucleic acids can be synthesized, or can be derived from any biological source, including any organism.

[0040] The terms "*CCL21*" and terms including "*CCL21*" (*e.g.*, *hCCL21*) are used

herein to refer to nucleic acids that encode a CCL21 polypeptide. Thus, the term "*CCL21*" refers to isolated nucleic acids of the present invention comprising: (a) a nucleotide sequence comprising the nucleotide sequence of SEQ ID NO:1; or (b) a nucleotide sequence substantially identical to SEQ ID NO:1.

[0041] The term "substantially identical", as used herein to describe a degree of similarity between nucleotide sequences, refers to two or more sequences that have at least about least 60%, preferably at least about 70%, more preferably at least about 80%, more preferably about 90% to about 99%, still more preferably about 95% to about 99%, and most preferably about 99% nucleotide identity, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection. Preferably, the substantial identity exists in nucleotide sequences of at least about 100 residues, more preferably in nucleotide sequences of at least about 150 residues, and most preferably in nucleotide sequences comprising a full length coding sequence. The term "full length" is used herein to refer to a complete open reading frame encoding a functional CCL21 polypeptide, as described further herein below. Methods for determining percent identity between two polypeptides are defined herein below under the heading "Nucleotide and Amino Acid Sequence Comparisons".

[0042] In one aspect, substantially identical sequences can be polymorphic sequences. The term "polymorphic" refers to the occurrence of two or more genetically determined alternative sequences or alleles in a population. An allelic difference can be as small as one base pair.

[0043] In another aspect, substantially identical sequences can comprise mutagenized sequences, including sequences comprising silent mutations. A mutation can comprise one or more residue changes, a deletion of residues, or an insertion of additional residues.

[0044] Another indication that two nucleotide sequences are substantially identical is that the two molecules hybridize specifically to or hybridize substantially to each other under stringent conditions. In the context of nucleic acid hybridization, two nucleic acid sequences being compared can be designated a "probe" and a "target." A "probe" is a reference nucleic acid molecule, and a "'target" is a test nucleic acid molecule, often found within a heterogeneous population of nucleic acid molecules. A "target sequence" is synonymous with a "test sequence."

[0045] A preferred nucleotide sequence employed for hybridization studies or assays

includes probe sequences that are complementary to or mimic at least an about 14 to 40 nucleotide sequence of a nucleic acid molecule of the present invention. Preferably, probes comprise 14 to 20 nucleotides, or even longer where desired, such as 30, 40, 50, 60, 100, 200, 300, or 500 nucleotides or up to the full length of any SEQ ID NO:1. Such fragments can be readily prepared by, for example, chemical synthesis of the fragment, by application of nucleic acid amplification technology, or by introducing selected sequences into recombinant vectors for recombinant production.

[0046] The phrase "hybridizing specifically to" refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex nucleic acid mixture (*e.g.*, total cellular DNA or RNA).

[0047] The phrase "hybridizing substantially to" refers to complementary hybridization between a probe nucleic acid molecule and a target nucleic acid molecule and embraces minor mismatches that can be accommodated by reducing the stringency of the hybridization media to achieve the desired hybridization.

[0048] "Stringent hybridization conditions" and "stringent hybridization wash conditions" in the context of nucleic acid hybridization experiments such as Southern and Northern blot analysis are both sequence- and environment-dependent. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) Laboratory Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Acid Probes, part I chapter 2, Elsevier, New York. Generally, highly stringent hybridization and wash conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. Typically, under "stringent conditions" a probe will hybridize specifically to its target subsequence, but to no other sequences.

[0049] The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Very stringent conditions are selected to be equal to the T_m for a particular probe. An example of stringent hybridization conditions for Southern or Northern Blot analysis of complementary nucleic acids having more than about 100 complementary residues is overnight hybridization in 50% formamide with 1 mg of heparin at 42°C. An example of highly stringent wash conditions is 15 minutes in 0.1X SSC at 65°C. An

example of stringent wash conditions is 15 minutes in 0.2X SSC buffer at 65°C. *See* Sambrook et al., eds (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York for a description of SSC buffer. Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. An example of medium stringency wash conditions for a duplex of more than about 100 nucleotides, is 15 minutes in 1X SSC at 45°C. An example of low stringency wash for a duplex of more than about 100 nucleotides, is 15 minutes in 4X to 6X SSC at 40°C. For short probes (*e.g.*, about 10 to 50 nucleotides), stringent conditions typically involve salt concentrations of less than about 1M Na^+ ion, typically about 0.01 to 1M Na^+ ion concentration (or other salts) at pH 7.0-8.3, and the temperature is typically at least about 30°C. Stringent conditions can also be achieved with the addition of destabilizing agents such as formamide. In general, a signal to noise ratio of 2-fold (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization.

[0050] The following are examples of hybridization and wash conditions that can be used to identify nucleotide sequences that are substantially identical to reference nucleotide sequences of the present invention: a probe nucleotide sequence preferably hybridizes to a target nucleotide sequence in 7% sodium dodecyl sulphate (SDS), 0.5M $NaPO_4$, 1mM EDTA at 50°C followed by washing in 2X SSC, 0.1% SDS at 50°C; more preferably, a probe and target sequence hybridize in 7% sodium dodecyl sulphate (SDS), 0.5M $NaPO_4$, 1mM EDTA at 50°C followed by washing in 1X SSC, 0.1% SDS at 50°C; more preferably, a probe and target sequence hybridize in 7% sodium dodecyl sulphate (SDS), 0.5M $NaPO_4$, 1mM EDTA at 50°C followed by washing in 0.5X SSC, 0.1% SDS at 50°C; more preferably, a probe and target sequence hybridize in 7% sodium dodecyl sulphate (SDS), 0.5M $NaPO_4$, 1mM EDTA at 50°C followed by washing in 0.1X SSC, 0.1% SDS at 50°C; more preferably, a probe and target sequence hybridize in 7% sodium dodecyl sulphate (SDS), 0.5M $NaPO_4$, 1mM EDTA at 50°C followed by washing in 0.1X SSC, 0.1% SDS at 65°C.

[0051] A further indication that two nucleic acid sequences are substantially identical is that proteins encoded by the nucleic acids are substantially identical, share an overall three-dimensional structure, or are biologically functional equivalents. These terms are defined further under the heading "CCL21 Polypeptides" herein below.

Nucleic acid molecules that do not hybridize to each other under stringent conditions are still substantially identical if the corresponding proteins are substantially identical. This can occur, for example, when two nucleotide sequences comprise conservatively substituted variants as permitted by the genetic code.

[0052] The term "conservatively substituted variants" refers to nucleic acid sequences having degenerate codon substitutions wherein the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues. *See* Batzer et al. (1991) *Nucleic Acids Res* 19:5081; Ohtsuka et al. (1985) *J Biol Chem* 260:2605-2608; and Rossolini et al. (1994) *Mol Cell Probes* 8:91-98.

[0053] The term "*CCL21*" also encompasses nucleic acids comprising subsequences and elongated sequences of a *CCL21* nucleic acid, including nucleic acids complementary to a *CCL21* nucleic acid, *CCL21* RNA molecules, and nucleic acids complementary to *CCL21* RNAs (cRNAs).

[0054] The term "subsequence" refers to a sequence of nucleic acids that comprises a part of a longer nucleic acid sequence. An exemplary subsequence is a probe, described herein above, or a primer. The term "primer" as used herein refers to a contiguous sequence comprising about 8 or more deoxyribonucleotides or ribonucleotides, preferably 10-20 nucleotides, and more preferably 20-30 nucleotides of a selected nucleic acid molecule. The primers of the invention encompass oligonucleotides of sufficient length and appropriate sequence so as to provide initiation of polymerization on a nucleic acid molecule of the present invention.

[0055] The term "elongated sequence" refers to an addition of nucleotides (or other analogous molecules) incorporated into the nucleic acid. For example, a polymerase (*e.g.*, a DNA polymerase) can add sequences at the 3' terminus of the nucleic acid molecule. In addition, the nucleotide sequence can be combined with other DNA sequences, such as promoters, promoter regions, enhancers, polyadenylation signals, intronic sequences, additional restriction enzyme sites, multiple cloning sites, and other coding segments.

[0056] The term "complementary sequences," as used herein, indicates two nucleotide sequences that comprise antiparallel nucleotide sequences capable of pairing with one another upon formation of hydrogen bonds between base pairs. As used herein, the term "complementary sequences" means nucleotide sequences which are substantially complementary, as can be assessed by the same nucleotide comparison methods set forth below, or is defined as being capable of hybridizing to

the nucleic acid segment in question under relatively stringent conditions such as those described herein. A particular example of a complementary nucleic acid segment is an antisense oligonucleotide.

[0057] The present invention also provides chimeric genes comprising the disclosed *CCL21* nucleic acids and recombinant *CCL21* nucleic acids. Thus, also included are constructs and vectors comprising *CCL21* nucleic acids.

[0058] The term "gene" refers broadly to any segment of DNA associated with a biological function. A gene encompasses sequences including but not limited to a coding sequence, a promoter region, a cis-regulatory sequence, a non-expressed DNA segment that is a specific recognition sequence for regulatory proteins, a non-expressed DNA segment that contributes to gene expression, a DNA segment designed to have desired parameters, or combinations thereof. A gene can be obtained by a variety of methods, including cloning from a biological sample, synthesis based on known or predicted sequence information, and recombinant derivation of an existing sequence.

[0059] The term "chimeric gene," as used herein, refers to a promoter region operatively linked to a *CCL21* sequence, including a *CCL21* cDNA, a *CCL21* nucleic acid encoding an antisense RNA molecule, a *CCL21* nucleic acid encoding an RNA molecule having tertiary structure (*e.g.*, a hairpin structure) or a *CCL21* nucleic acid encoding a double-stranded RNA molecule. The term "chimeric gene" also refers to a *CCL21* promoter region operatively linked to a heterologous sequence.

[0060] The term "operatively linked", as used herein, refers to a functional combination between a promoter region and a nucleotide sequence such that the transcription of the nucleotide sequence is controlled and regulated by the promoter region. Techniques for operatively linking a promoter region to a nucleotide sequence are known in the art.

[0061] The term "recombinant" generally refers to an isolated nucleic acid that is replicable in a non-native environment. Thus, a recombinant nucleic acid can comprise a non-replicable nucleic acid in combination with additional nucleic acids, for example vector nucleic acids, that enable its replication in a host cell.

[0062] The term "vector" is used herein to refer to a nucleic acid molecule having nucleotide sequences that enable its replication in a host cell. A vector can also include nucleotide sequences to permit ligation of nucleotide sequences within the vector, wherein such nucleotide sequences are also replicated in a host cell.

Representative vectors include plasmids, cosmids, and viral vectors. A vector can also mediate recombinant production of a CCL21 polypeptide, as described further herein below.

[0063] The term "construct", as used herein to describe a type of construct comprising an expression construct, refers to a vector further comprising a nucleotide sequence operatively inserted with the vector, such that the nucleotide sequence is recombinantly expressed.

[0064] The terms "recombinantly expressed" or "recombinantly produced" are used interchangeably to refer generally to the process by which a polypeptide encoded by a recombinant nucleic acid is produced.

[0065] Thus, preferably recombinant *CCL21* nucleic acids comprise heterologous nucleic acids. The term "heterologous nucleic acids" refers to a sequence that originates from a source foreign to an intended host cell or, if from the same source, is modified from its original form. A heterologous nucleic acid in a host cell can comprise a nucleic acid that is endogenous to the particular host cell but has been modified, for example by mutagenesis or by isolation from native cis-regulatory sequences. A heterologous nucleic acid also includes non-naturally occurring multiple copies of a native nucleotide sequence. A heterologous nucleic acid can also comprise a nucleic acid that is incorporated into a host cell's nucleic acids at a position wherein such nucleic acids are not ordinarily found.

[0066] Nucleic acids of the present invention can be cloned, synthesized, altered, mutagenized, or combinations thereof. Standard recombinant DNA and molecular cloning techniques used to isolate nucleic acids are known in the art. Site-specific mutagenesis to create base pair changes, deletions, or small insertions are also known in the art. *See e.g.,* Sambrook et al. (eds.) (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Silhavy et al. (1984) Experiments with Gene Fusions. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Glover & Hames (1995) DNA Cloning: A Practical Approach, 2nd ed. IRL Press at Oxford University Press, Oxford / New York; Ausubel (ed.) (1995) Short Protocols in Molecular Biology, 3rd ed. Wiley, New York.

A.2 CCL21 Polypeptides

[0067] The present invention provides novel CCL21 polypeptides, a representative embodiment of which is set forth as SEQ ID NO:2. Preferably, an isolated CCL21

polypeptide of the present invention comprises a recombinantly expressed CCL21 polypeptide. Also preferably, isolated CCL21 polypeptides comprise functional CCL21 polypeptides.

[0068] Thus, novel CCL21 polypeptides useful in the methods of the present invention comprise: (a) a polypeptide of SEQ ID NO:2; (b) a polypeptide substantially identical to SEQ ID NO:2; (c) a polypeptide encoded by a nucleic acid molecule of SEQ ID NO:1; or (d)a polypeptide encoded by a nucleic acid molecule substantially identical to SEQ ID NO:1. A CCL21 polypeptide can also comprise: (a) an isolated nucleic acid molecule encoding a polypeptide of SEQ ID NO:2; (b) an isolated nucleic molecule of SEQ ID NO:1; (c) an isolated nucleic acid molecule which hybridizes to a CCL21 nucleic acid sequence under wash stringency conditions represented by a wash solution having less than about 200 mM salt concentration and a wash temperature of greater than about 45°C, and which encodes a CCL21 polypeptide; and (d) an isolated nucleic acid molecule differing by at least one functionally equivalent codon from the isolated nucleic acid molecule of one of (a), (b), and (c) above in nucleic acid sequence due to the degeneracy of the genetic code, and which encodes a CCL21 polypeptide encoded by the isolated nucleic acid of one of (a), (b), and (c) above.

[0069] The term "substantially identical", as used herein to describe a level of similarity between CCL21 and a protein substantially identical to a CCL21 protein, refers to a sequence that is at least about 35% identical to SEQ ID NO:2, when compared over the full length of a CCL21 protein. Preferably, a protein substantially identical to a CCL21 protein comprises an amino acid sequence that is at least about 35% to about 45% identical to SEQ ID NO:2, more preferably at least about 45% to about 55% identical to SEQ ID NO:2, even more preferably at least about 55% to about 65% identical to SEQ ID NO:2, still more preferably at least about 65% to about 75% identical to SEQ ID NO:2, still more preferably at least about 75% to about 85% identical to SEQ ID NO:2, still more preferably at least about 85% to about 95% identical to SEQ ID NO:2, and still more preferably at least about 95% to about 99% identical to SEQ ID NO:2 when compared over the full length of a CCL21 polypeptide. The term "full length" refers to a functional CCL21 polypeptide, as described further herein below. Methods for determining percent identity between two polypeptides are also defined herein below under the heading "Nucleotide and Amino Acid Sequence Comparisons".

[0070] The term "substantially identical," when used to describe polypeptides, also encompasses two or more polypeptides sharing a conserved three-dimensional structure. Computational methods can be used to compare structural representations, and structural models can be generated and easily tuned to identify similarities around important active sites or ligand binding sites. *See* Saqi et al. (1999) *Bioinformatics* 15:521-522; Barton (1998) *Acta Crystallogr D Biol Crystallogr* 54:1139-1146; Henikoff et al. (2000) *Electrophoresis* 21:1700-1706; and Huang et al. (2000) *Pac Symp Biocomput*:230-241.

[0071] Substantially identical proteins also include proteins comprising amino acids that are functionally equivalent to amino acids of SEQ ID NO:2. The term "functionally equivalent" in the context of amino acids is known in the art and is based on the relative similarity of the amino acid side-chain substituents. *See* Henikoff & Henikoff (2000) *Adv Protein Chem* 54:73-97. Relevant factors for consideration include side-chain hydrophobicity, hydrophilicity, charge, and size. For example, arginine, lysine, and histidine are all positively charged residues; that alanine, glycine, and serine are all of similar size; and that phenylalanine, tryptophan, and tyrosine all have a generally similar shape. By this analysis, described further herein below, arginine, lysine, and histidine; alanine, glycine, and serine; and phenylalanine, tryptophan, and tyrosine; are defined herein as biologically functional equivalents.

[0072] In making biologically functional equivalent amino acid substitutions, the hydropathic index of amino acids can be considered. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics, these are: isoleucine (+ 4.5); valine (+ 4.2); leucine (+ 3.8); phenylalanine (+ 2.8); cysteine (+ 2.5); methionine (+ 1.9); alanine (+ 1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

[0073] The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is generally understood in the art (Kyte et al., 1982). It is known that certain amino acids can be substituted for other amino acids having a similar hydropathic index or score and still retain a similar biological activity. In making changes based upon the hydropathic index, the substitution of amino acids whose hydropathic indices are within ±2 of the original value is preferred, those

which are within ±1 of the original value are particularly preferred, and those within ±0.5 of the original value are even more particularly preferred.

[0074] It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Patent No. 4,554,101 describes that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity and antigenicity, *e.g.*, with a biological property of the protein. It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent protein.

[0075] As detailed in U.S. Patent No. 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+ 3.0); lysine (+ 3.0); aspartate (+ 3.0±1); glutamate (+ 3.0±1); serine (+ 0.3); asparagine (+ 0.2); glutamine (+ 0.2); glycine (0); threonine (-0.4); proline (-0.5±1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

[0076] In making changes based upon similar hydrophilicity values, the substitution of amino acids whose hydrophilicity values are within ±2 of the original value is preferred, those which are within ±1 of the original value are particularly preferred, and those within ±0.5 of the original value are even more particularly preferred.

[0077] The term "substantially identical" also encompasses polypeptides that are biologically functional equivalents of a CCL21 polypeptide. The term "functional" includes an activity of an CCL21 polypeptide, for example promoting migration of dendritic cells and other lymphocytes, enhancing immunogenicity of an antigen, inhibiting cancer growth, and promoting cancer resistance. Representative methods for assessing CCL21 activity are described in the Examples.

[0078] The present invention also provides functional fragments of a CCL21 polypeptide. Such functional portion need not comprise all or substantially all of the amino acid sequence of a native CCL21 gene product.

[0079] The present invention also includes functional polypeptide sequences that are longer sequences than that of a native CCL21 polypeptide. For example, one or more amino acids can be added to the N-terminus or C-terminus of a CCL21 polypeptide. Such additional amino acids can be employed in a variety of applications, including but not limited to purification applications. Methods of preparing elongated proteins are known in the art.

[0080]CCL21 polypeptides can be produced and characterized using a variety of standard techniques that are known to the skilled artisan. *See e.g.*, Schröder & Lübke (1965) The Peptides. Academic Press, New York; Schneider & Eberle (1993) Peptides, 1992: Proceedings of the Twenty-Second European Peptide Symposium, September 13-19, 1992, Interlaken, Switzerland. Escom, Leiden; Bodanszky (1993) Principles of Peptide Synthesis, 2nd rev. ed. Springer-Verlag, Berlin; New York; and Ausubel (ed.) (1995) Short Protocols in Molecular Biology, 3rd ed. Wiley, New York. A representative method for producing CCL21 in baculovirus is described in Example 1.

A.3. Nucleotide and Amino Acid Sequence Comparisons

[0081]The terms "identical" or "percent identity" in the context of two or more nucleotide or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence, as measured using one of the sequence comparison algorithms disclosed herein or by visual inspection.

[0082]The term "substantially identical" in regards to a nucleotide or polypeptide sequence means that a particular sequence varies from the sequence of a naturally occurring sequence by one or more deletions, substitutions, or additions, the net effect of which is to retain biological function of a CCL21 nucleic acid or a CCL21 polypeptide.

[0083]For comparison of two or more sequences, typically one sequence acts as a reference sequence to which one or more test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer program, subsequence coordinates are designated if necessary, and sequence algorithm program parameters are selected. The sequence comparison algorithm then calculates the percent sequence identity for the designated test sequence(s) relative to the reference sequence, based on the selected program parameters.

[0084]Optimal alignment of sequences for comparison can be conducted, for example, by the local homology algorithm of Smith & Waterman (1981) *Adv Appl Math* 2:482-489, by the homology alignment algorithm of Needleman & Wunsch (1970) *J Mol Biol* 48:443-453, by the search for similarity method of Pearson & Lipman (1988) *Proc Natl Acad Sci USA* 85:2444-2448, by computerized

implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, Madison, Wisconsin), or by visual inspection. *See* generally, Ausubel (ed.) (1995) Short Protocols in Molecular Biology, 3rd ed. Wiley, New York.

[0085] A preferred algorithm for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul et al. (1990) *J Mol Biol* 215:403-410. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold. These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when the cumulative alignment score falls off by the quantity X from its maximum achieved value, the cumulative score goes to zero or below due to the accumulation of one or more negative-scoring residue alignments, or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength W=11, an expectation E=10, a cutoff of 100, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix. *See* Henikoff & Henikoff (1992) *Proc Natl Acad Sci U S A* 89:10915-10919.

[0086] In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences. *See e.g.*, Karlin & Altschul (1993) *Proc Natl Acad Sci USA* 90:5873-5877. One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)),

which provides an indication of the probability by which a match between two nucleotide or amino acid sequences that would occur by chance. For example, a test nucleic acid sequence is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid sequence to the reference nucleic acid sequence is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

B. Adjuvant Activity of Non-Pathogenic Viruses

[0087] In the course of developing cancer immunotherapies that employ Secondary Lymphoid Tissue Chemokine (referred to herein as "CCL21;" also known in the art as SLC, Exodus-2, and 6C-kine), as described in Examples 1-6, the inventors of the subject disclosure came to the surprising discovery that non-pathogenic viruses are effective adjuvants. *See* Examples 9-10. The term "adjuvant" is meant to refer to a molecule having the ability to enhance or otherwise modulate the response of a vertebrate subject's immune system to an antigen. For example, baculovirus activates dendritic cell maturation and cytolytic T cell (CTL) responses both *in vitro* and *in vivo*. *See* Examples 9-10. *See also* Gronowski et al. (1999) *J Virol* 73:9944-51.

[0088] The present invention further discloses that the anti-tumor effects of baculovirus-expressed CCL21 are attributable, at least in part, to baculoviral contaminants in the preparation. When such contaminant was removed, the anti-tumor of the CCL21 preparation was substantially abolished. *See* Example 7. Thus, the present invention provides for administering CCL21 in combination with an adjuvant to thereby inhibit and/or prevent tumor growth *in vivo*.

[0089] The methods of the present invention are useful for treating a subject in need of anti-cancer therapy, including inhibition of cancer growth, inhibition of cancer metastasis, and cancer resistance, via administering to a subject CCL21 and an adjuvant, preferably an adjuvant comprising a non-pathogenic virus. Significantly, the combination immunotherapy disclosed herein does not rely on identification of tumor-specific antigens. Rather, the disclosed methods are broadly applicable and are efficacious in multiple tumor types. *See* Examples 2-6.

[0090] Adjuvants are used to improve the activity of both heterologous and endogenous antigens by modulating immune responses, including (1) stimulating humoral and cell mediated immunity; (2) eliciting cytokine and chemokine production

by antigen presenting cells; and (3) controlling the type of acquired immune response that is induced. *See* O'Hagan et al. (2001) *Biomol Eng* 18:69-85 and Yip (1999) *J Immunol* 162:3942-3949.

[0091] Any adjuvant known in the art is suitable for use in the accordance with the methods of the present invention, including but not limited to MF59 (oil/water emulsion), alum, CpG (hypermethylated DNA motif), L-prolyl-L-leucyl-glycinamide (PLG) particles, virus and particularly a non-pathogenic virus, endotoxins, and chemokines with activities not shared by CCL21.

[0092] In a preferred embodiment of the invention, an adjuvant comprises a non-pathogenic virus, including a live virus, inactivated virus, virus particles, viral occlusion bodies, viral components, and combinations thereof.

[0093] The term "live virus" refers to a virus whose infectivity is similar or identical to a native virus. In particular, a live virus can infect its native host cells.

[0094] The term "inactivated virus" refers to a virus that is incapable of replication in a native host cell, as described further herein below. For example, a non-pathogenic virus, which is incapable of replication in a mammalian host cell, is similarly incapable of replication in it native host cell upon being inactivated. Inactivated viruses are preferred to minimize safety concerns regarding administration of live viruses.

[0095] The term "virus particle" refers to a virus that has been constructed, or modified from its native form, whereby it is unable to replicate in naturally occurring host cells. Methods for preparing virus particles are known in the art. The structural and functional integrity of virus-like particles can be assessed by electron microscopy, immunogenicity analyses, and standard plaque assays.

[0096] U.S. Patent No. 5,750,383 discloses methods for preparing baculovirus particles using a marker-rescue system. The method employs a genetically modified baculovirus, which lacks a gene essential for viral replication (*e.g.*, gp64), and which is propagated in cells that complement the genetic deficiency.

[0097] The term "viral occlusion body" refers to a structure comprising a multiplicity of viral particles embedded within a virus-encoded proteinaceous crystal. Upon dissolution of the protein crystal, the multiplicity of viral particles is released, and each viral particles is capable of subsequent infection of a host cell.

[0098] Production of viruses, and in particular baculoviruses, is accomplished using techniques well known in the art. Cloned cell lines are provided in a culture medium *in vitro*, inoculated with virus, and incubated for a sufficient time and under conditions effective to allow viral production. Culture conditions, including cell density, multiplicity of infection, time, temperature, media, *etc.* are not critical and can be readily determined by a practitioner skilled in the art.

[0099] Representative methods for baculovirus production are described in Example 1, which employ *Spodoptera frugiperda* (Sf) cells. Additional representative host cells and amplification methods are described in U.S. Patent Nos. 5,405,770 (*Heliothis subflexa* cell line) and 6,379,958 (*Spodoptera frugiperda* cell lines, which show improved baculovirus production).

[0100] Following incubation, the viral agents so produced are recovered by techniques conventional in the art, including polyethylene glycol (PEG) precipitation, ultracentrifugation, and chromatographic purification, such as use of an ion exchange resin, size exclusion chromatography, affinity chromatography, or combinations thereof. *See* U.S. Patent Application Publication No. 2002/0015945 (chromatographic purification); U.S. Patent No. 6,194,192 (viral adsorption to sulfated-fucose-containing polysaccharide(s)).

[0101] The term "viral component," as used herein, refers to a molecule that is derived from a non-pathogenic virus and that retains adjuvant activity of the parent live virus. Preferably, a viral component comprises adjuvant activity that is similar in magnitude and specificity of response when compared to that elicited by the parent live virus from which it was derived. The term "viral component" encompasses any biological component of a virus, including a protein, a peptide, a nucleic acid, a lipid, a carbohydrate, any other bioactive molecule of a virus, and combinations thereof.

[0102] For example, a viral component can comprise a viral capsid protein or a DNA-associated protein of the viral nucleoprotein core. Representative baculoviral capsid proteins are described by Pearson et al. (1988) *Virology* 167:407-13; by Summers & Smith (1978) *Virology* 84:390-402; by Thiem & Miller (1989) *J Virol* 63:2008-18; and by Vialard & Richardson (1993) *J Virol* 67:5859-66. Representative baculoviral DNA-associated proteins are described by Tweeten et al. (1980) *J Virol* 33:866-876; by Wilson et al. (1987) *J Virol* 61:661-6; and by Rohrmann (1992) *J Gen Virol* 73 (Pt 4):749-61.

[0103] A viral component can also comprise proteins and carbohydrates found in viral occlusion bodies, including the occlusion body matrix and the calyx outer layer found in mature occlusion bodies. Representative baculovirus occlusion body proteins include polyhedron and calyx.

[0104] Following a review of the disclosure herein, which provides that non-pathogenic viruses have potent anti-tumor activity, a skilled artisan could readily identify, purify or otherwise prepare, and administer viral components to recapitulate the anti-tumor activity of the parent live virus. For example, as one approach, U.S. Patent No. 6,001,806 discloses biochemical methods for fractionating baculovirus-infected insect cells, and then using the eluate fractions in assays to identify a glycoprotein that mimics the anti-viral activity previously recognized in the parent live virus.

[0105] In addition, viral proteins and nucleic acids are readily prepared using recombinant methods known in the art and can be similarly tested for anti-cancer activity. For example, viral nucleic acids can be cloned, synthesized, altered, mutagenized, or combinations thereof. Standard recombinant DNA and molecular cloning techniques used to isolate nucleic acids can be found, for example, in Sambrook et al. (eds.) (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Silhavy et al. (1984) Experiments with Gene Fusions, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Glover & Hames (1995) DNA Cloning: A Practical Approach, 2nd ed. IRL Press at Oxford University Press, Oxford/New York; and Ausubel (ed.) (1995) Short Protocols in Molecular Biology, 3rd ed. Wiley, New York. Recombinantly produced polypeptides can also be purified and characterized using a variety of standard techniques that are known to the skilled artisan. *See e.g.*, Schröder & Lübke (1965) The Peptides. Academic Press, New York; Schneider & Eberle (1993) Peptides, 1992: Proceedings of the Twenty-Second European Peptide Symposium, September 13-19, 1992, Interlaken, Switzerland. Escom, Leiden; Bodanszky (1993) Principles of Peptide Synthesis, 2nd rev. ed. Springer-Verlag, Berlin/New York; and Ausubel (ed.) (1995) Short Protocols in Molecular Biology, 3rd ed. Wiley, New York.

[0106] In particular, the complete sequence of AcNPV is known (Kool and Vlak, 1993), and thus a systematic analysis of all AcNPV proteins can be readily

performed using known methods for recombinant expression in combination with assays for adjuvant activity.

[0107] The term "non-pathogenic," as used herein to describe a virus, refers to a virus that is not infectious in a mammalian host to be treated with the virus, and more preferably, a non-pathogenic virus is not infectious in any mammalian host. Still more preferably, a non-pathogenic virus is not infectious in a human host.

[0108] The term "non-pathogenic," as used herein to describe a virus, refers to a virus that does not replicate in a mammalian host to be treated with the virus, and more preferably, a non-pathogenic virus does not replicate in any mammalian host. Still more preferably, a non-pathogenic virus does not replicate in a human host. As described further herein below, non-pathogenicity does not preclude entry into a mammalian cell.

[0109] A non-pathogenic virus, for example baculovirus, can also be transcriptionally silent in mammalian host cells. Thus, a non-pathogenic virus is a type of virus that is specifically excluded from current gene therapy methods, as heterologous genes are also not expressed.

[0110] Non-pathogenic viruses to be used in accordance with the methods of the present invention include but are not limited to insect-specific viruses, amphibian-specific viruses, and plant-specific viruses. Representative viruses useful in the methods disclosed herein include viruses of the family Baculoviridae (*e.g.*, nucleopolyhedroviruses (NPV) such as *Autographa californica* NPV, and granulosis viruses (GV) such as *Trichoplusia ni* GV), Polydnaviridae (*e.g.*, ichnoviruses such as *Campoletis sonorensis* virus, and bracoviruses such as *Cotesia melanoscela* virus), Ascoviruses, Tetraviridae, and Nodaviridae (*e.g.*, nodaviruses such as *Nodamura* virus and Flock House Virus). Each of these non-pathogenic viruses are found solely or primarily in insects. *See* Fields et al., eds. (1996) Virology, Lippincott-Rave Publishers, Philadelphia, Pennsylvania.

[0111] In one embodiment of the invention, a non-pathogenic virus comprises a baculovirus. As described in Example 2-3 and 7, the present invention provides that CCL21, when administered in combination with baculovirus, is a potent inhibitor of tumor growth and can promote complete tumor remission. The present invention further provides that baculovirus can be used to inhibit tumor metastasis and to

promote resistance to tumor re-challenge, as described in Examples 5-7. In accordance with the definition provided herein above, the term "baculovirus" encompasses baculovirus particles and baculovirus components.

[0112] The host specificity of baculovirus has been thoroughly studied. Although baculovirus is known to infect over 30 species of *Lepidoptera*, it does not replicate in other insect cells or in any of the over 35 mammalian cell lines studied. *See* Tjia et al. (1983) *Virology* 125:107-17; Volkman & Goldsmith (1983) *Appl Environ Microbiol* 45:1085-1093; and McIntosh & Shamy (1980) *Intervirology* 13:331-41. Baculovirus does, however, enter mammalian cells and viral DNA can be detected in the host cell nucleus. *See* Groner et al. (1984) *Intervirology* 21:203-9; Tjia et al. (1983) *Virology* 125:107-17; and Volkman & Goldsmith (1983) *Appl Environ Microbiol* 45:1085-93.

[0113] The term "non-pathogenic" further encompasses viruses, which are pathogenic in their native form, and which have been modified to be non-pathogenic. Such modification can include genetic modification (*e.g.*, disruption of a gene that is essential for viral replication, as described herein above for the baculovirus gp64 gene; and/or disruption of a viral promoter to render it transcriptionally inactive in the host species). For example, the species-specific pathogenicity of baculovirus is due in part to silence of the baculovirus promoter in species other than *Lepidoptera*. When a heterologous promoter is inserted into baculovirus genome, the modified virus becomes capable of gene expression in non-*Lepidopteran* cell lines, including various mammalian cell lines. *See* Boyce & Bucher (1996) *Proc Natl Acad Sci USA* 93:2348-52; Carbonell et al. (1985) *J Virol* 56: 153-60; Carbonell & Miller (1987) *Appl Environ Microbiol* 53:1412-7; and Hofmann et al. (1995) *Proc Natl Acad Sci USA* 92:10099-103. A viral promoter that is initially active in mammalian cells could be similarly modified to the opposite result, whereby it is no longer pathogenic in mammalian species. Methods for site-specific mutagenesis to create base pair changes, deletions, or small insertions are also known in the art, for example as described in the references noted herein above.

[0114] Modified viruses, as well as unmodified viruses that are suspected to be non-pathogenic, can be readily assayed for non-pathogenicity using methods for determining viral infectivity and replication known in the art. Representative methods can be found, for example, in Tjia et al. (1983) *Virology* 125:107-17; Volkman &

Goldsmith (1983) *Appl Environ Microbiol* 45:1085-93; McIntosh & Shamy (1980) *Intervirology* 13:331-41; and U.S. Patent No. 6,248,514, among other places.

[0115] The present invention also provides non-pathogenic viruses having adjuvant activity, including live viruses, inactive viruses, viral particles, viral occlusion bodies, and viral components. Also provided are methods for selecting a non-pathogenic virus useful in the therapeutic methods described herein. To select a non-pathogenic virus having adjuvant activity, candidate non-pathogenic viruses can be tested for ability to enhance immunogenicity of an antigen. Immunogenicity can be determined by, for example, detecting T cell-mediated responses. Representative methods for measuring T cell responses include *in vitro* cytotoxicity assays or *in vivo* delayed-type hypersensitivity assays. In particular, CCL21 in combination with a non-pathogenic virus can induce *in vitro* cytotoxicity of tumor cells by PBMCs, and this activity correlates with anti-tumor activity upon *in vivo* administration. Immunogenicity can also be assessed by detection of antigen-specific antibodies in a subject's serum, and/or by a demonstration of protective effects of antisera or immune cells specific for the antigen. Preferably, a non-pathogenic virus enhances immunogenicity of an antigen by at least about 2-fold, more preferably at least about 5-fold, and still more preferably at least about 10-fold.

[0116] Non-pathogenic viruses provided by the invention also are preferably inactivated, as described further herein below. Non-pathogenic viruses, which show adjuvant activity, can be subjected to any one of a variety of inactivation methods to render the virus incapable of infecting its native host cell. Using the assays disclosed herein, a skilled artisan can select an inactivation method that preserves adjuvant activity of the virus. Thus, preferred inactivation methods permit viral entry into host cells, and disrupt transcription and/or replication of the viral genome. In one embodiment of the invention, a virus is genetically modified such that it is capable of cellular entry, but is unable to undergo normal transcription and/or replication.

C. Therapeutic Applications

[0117] The present invention provides methods for treating a cancer-bearing mammalian subject via administering CCL21 in combination with an adjuvant. The disclosed methods are useful for anti-cancer therapy, including cancer prevention. In particular, co-administration of CCL21 and an adjuvant promotes an adaptive

immune response that mediates cancer resistance. As noted herein above, the adjuvant is preferably a non-pathogenic virus, such as baculovrius.

[0118] The term "cancer growth" generally refers to any one of a number of indices that suggest change within the cancer to a more developed form. Thus, indices for measuring an inhibition of cancer growth include but are not limited to a decrease in cancer cell survival, a decrease in tumor volume or morphology (for example, as determined using computed tomographic (CT), sonography, or other imaging method), a delayed tumor growth, a destruction of tumor vasculature, improved performance in delayed hypersensitivity skin test, an increase in the activity of cytolytic T-lymphocytes, and a decrease in levels of tumor-specific antigens.

[0119] The term "delayed tumor growth" refers to a decrease in a duration of time required for a tumor to grow a specified amount. For example, treatment can delay the time required for a tumor to increase in volume 3-fold relative to an initial day of measurement (day 0) or the time required to grow to 1 cm^3.

[0120] The term "cancer resistance" refers to a improved capacity of a subject to resist cancer growth, in particular growth of a cancer already had. Alternatively stated, the term "cancer resistance" refers to a decreased propensity for cancer growth in a subject.

[0121] The term "subject" as used herein includes any mammalian species. More particularly, the methods of the present invention are contemplated for the treatment of cancers in mammals such as humans, as well as those mammals of importance due to being endangered, of economical importance and/or social importance to humans.

[0122] The term "cancer" generally refers to tumors, including both primary and metastasized solid tumors. The term "tumor" encompasses solid tumors and carcinomas of any tissue in a subject, including but not limited to breast; colon; rectum; lung; oropharynx; hypopharynx; esophagus; stomach; pancreas; liver; gallbladder; bile ducts; small intestine; urinary tract including kidney, bladder and urothelium; female genital tract including cervix, uterus, ovaries (*e.g.*, choriocarcinoma and gestational trophoblastic disease); male genital tract including prostate, seminal vesicles, testes and germ cell tumors; endocrine glands including thyroid, adrenal, and pituitary; skin (*e.g.*, hemangiomas and melanomas), bone or

soft tissues; blood vessels (*e.g.*, Kaposi's sarcoma); brain, nerves, eyes, and meninges (*e.g.*, astrocytomas, gliomas, glioblastomas, retinoblastomas, neuromas, neuroblastomas, Schwannomas and meningiomas).

[0123] The term "tumor" also encompasses solid tumors arising from hematopoietic malignancies such as leukemias, including chloromas, plasmacytomas, plaques and tumors of mycosis fungoides and cutaneous T-cell lymphoma/leukemia, and lymphomas including both Hodgkin's and non-Hodgkin's lymphomas.

[0124] The term "cancer," as used herein, also encompasses non-neoplastic proliferative disorders. Thus, the methods of the present invention are contemplated for the treatment or prevention of hyperplasia, metaplasia, or most particularly, dysplasia (for review of such abnormal growth conditions, *see* Robbins & Angell (1976) Basic Pathology, 2d Ed., pp. 68-79, W. B. Saunders Co., Philadelphia, Pennsylvania).

[0125] Hyperplasia is a form of controlled cell proliferation involving an increase in cell number in a tissue or organ, without significant alteration in structure or function. As but one example, endometrial hyperplasia often precedes endometrial cancer. Metaplasia is a form of controlled cell growth in which one type of adult or fully differentiated cell substitutes for another type of adult cell. Metaplasia can occur in epithelial or connective tissue cells. Atypical metaplasia involves a somewhat disorderly metaplastic epithelium. Dysplasia is frequently a forerunner of cancer, and is found mainly in the epithelia; it is the most disorderly form of non-neoplastic cell growth, involving a loss in individual cell uniformity and in the architectural orientation of cells. Dysplastic cells often have abnormally large, deeply stained nuclei, and exhibit pleomorphism. Dysplasia characteristically occurs where there exists chronic irritation or inflammation, and is often found in the cervix, respiratory passages, oral cavity, and gall bladder. Although preneoplastic lesions can progress to neoplasia, they can also remain stable for long periods and can even regress, particularly if the inciting agent is removed or if the lesion succumbs to an immunological attack by its host.

[0126] Thus, administration of CCL21 in combination with a non-pathogenic virus to a subject, as disclosed herein, can elicit an innate anti-cancer immune response or an adaptive, cancer-specific immune response. The term "immune system" includes all the cells, tissues, systems, structures and processes, including non-specific and

specific categories, that provide a defense against cells comprising antigenic molecules, including but not limited to tumors, pathogens, and self-reactive cells. Thus, an immune response can comprise an innate immune response, an adaptive immune response, or a combination thereof.

[0127] The term "innate immune system" includes phagocytic cells such as neutrophils, monocytes, tissue macrophages, Kupffer cells, alveolar macrophages, dendritic cells, and microglia. The innate immune system mediates non-specific immune responses. The innate immune system plays an important role in initiating and guiding responses of the adaptive immune system. *See e.g.*, Janeway (1989) *Cold Spring Harb Symp Quant Biol* 54:1-13; Romagnani (1992) *Immunol Today* 13:379-381; Fearon & Locksley (1996) *Science* 272:50-53; and Fearon (1997) *Nature* 388:323-324. An innate response can comprise, for example, dendritic cell maturation, macrophage activation, cytokine or chemokine secretion, and/or activation of NFκB signaling.

[0128] The term "adaptive immune system" refers to the cells and tissues that impart specific immunity within a host. Included among these cells are natural killer (NK) cells and lymphocytes (*e.g.*, B cell lymphocytes and T cell lymphocytes). The term "adaptive immune system" also includes antibody-producing cells and the antibodies produced by the antibody-producing cells.

[0129] The term "adaptive immune response" refers to a specific response to an antigen include humoral immune responses (*e.g.*, production of antigen-specific antibodies) and cell-mediated immune responses (*.e.g.*, lymphocyte proliferation), as defined herein below. An adaptive immune response can further comprise systemic immunity and humoral immunity.

[0130] The terms "cell-mediated immunity" and "cell-mediated immune response" are meant to refer to the immunological defense provided by lymphocytes, such as that defense provided by T cell lymphocytes when they come into close proximity to their victim cells. A cell-mediated immune response also comprises lymphocyte proliferation. When "lymphocyte proliferation" is measured, the ability of lymphocytes to proliferate in response to specific antigen is measured. Lymphocyte proliferation is meant to refer to B cell, T-helper cell or CTL cell proliferation.

[0131] The term "CTL response" is meant to refer to the ability of an antigen-specific cell to lyse and kill a cell expressing the specific antigen. As described herein below, standard, art-recognized CTL assays are performed to measure CTL activity.

[0132] The term "systemic immune response" is meant to refer to an immune response in the lymph node-, spleen-, or gut-associated lymphoid tissues wherein cells, such as B lymphocytes, of the immune system are developed. For example, a systemic immune response can comprise the production of serum immunoglobulins (IgGs). Further, systemic immune response refers to antigen-specific antibodies circulating in the blood stream and antigen-specific cells in lymphoid tissue in systemic compartments such as the spleen and lymph nodes.

[0133] The terms "humoral immunity" or "humoral immune response" are meant to refer to the form of acquired immunity in which antibody molecules are secreted in response to antigenic stimulation.

[0134] The term "cancer-specific," as used herein to describe an adaptive immune response, refers to a cell-mediated or humoral immune response in a subject, wherein the response is directed specifically to a cancer previously present in the subject. Given that innate and adaptive immune responses involve unique immune cell types, one would not expect that methods for eliciting an innate immune response could also elicit an adaptive immune response. More preferably, administration of a non-pathogenic virus to a subject elicits both an innate immune response and an adaptive immune response.

[0135] In other preferred embodiments of the present invention, the methods disclosed herein for administration of non-pathogenic viruses can be combined with one or more other cancer therapies. For example, a tumor or abnormal cell growth can be surgically removed before or after administration of a non-pathogenic virus. Similarly, a non-pathogenic virus of the invention can be co-administered or co-formulated with additional agents, for example anti-angiogenic and/or additional immunomodulatory agents. Representative agents that can be used in conjunction with a non-pathogenic virus include but are not limited to methoxtrexate, tamoxifen, nelandron, nilutamide, adriamycin, 5-fluorouracil (5FU), cytokines such interferon alpha (IFN-α), interferon gamma (IFN-γ), interleukin 2 (IL2), interleukin 4 (IL4), interleukin 6 (IL6), and tumor necrosis factor (TNF).

[0136] The present invention further relates to methods and compositions useful for inducing cytotoxic T-cell mediated responses in mammalian subjects, including humans.

More particularly, the present invention relates to the use of a non-pathogenic virus for inducing cytotoxic T-cell mediated responses. Thus, the present invention provides methods for preparing antigen formulations comprising a non-pathogenic virus and a an antigen. The term "antigen" refers to a substance that activates lymphocytes (positively or negatively) by interacting with T cell or B cell receptors. Positive activation leads to immune responsiveness, and negative activation leads to immune tolerance. An antigen can comprise a protein, a carbohydrate, a lipid, a nucleic acid, or combinations thereof. An antigen can comprise a heterologous (*e.g.*, an antigen that is typically not found in a host subject) or an autologous antigen (self antigen).

[0137] Also provided are methods for using the disclosed antigen formulations as therapeutic and/or prophylactic agents. For example, such antigen formulations can be administered to a mammalian subject for the treatment of vital diseases in which a CTL response is important, for example, in the treatment of HIV infection or influenza; it can also be extended to use in treatment or prevention of bacterial infections, parasitic infections, and the like.

D. Therapeutic Compositions and Methods

[0138] The present invention further provides pharmaceutical compositions for anti-cancer therapy comprising: (a) an effective amount of CCL21; and (b) an effective amount of adjuvant. A composition comprising a *CCL21* nucleic acid and a non-pathogenic virus as adjuvant is prepared by admixing the nucleic acid and virus. Thus, the present invention is not limited to compositions comprising a CCL21-expressing viral vector. Rather, the present invention teaches improved chemokine therapies when merely admixed with an adjuvant comprising a non-pathogenic virus. Indeed, non-pathogenic viruses are not suitable vectors for gene therapy because they are typically transcriptionally silent in a mammalian host.

D.1. Virus Inactivation

[0139] Preferably, live non-pathogenic viruses employed as adjuvants in the methods of the present invention are inactivated prior to administration to a subject. Non-

pathogenic viruses, as defined herein above, are incapable of replication in a mammalian host. Inactivation, which renders the virus non-replicative in its native host cell, is preferred as an additional safety measure.

[0140] Viral inactivation can be accomplished by any suitable means, including but not limited to destruction of lipid or protein components of a viral coat, modification such that the virus is unrecognizable to a target cell, destruction of viral nucleic acid, and/or rendering of the virus as irreplicable. Representative methods for viral inactivation include but not limited to pasteurization, treatment with detergents (*e.g.*, Triton-X100®), alkylation with binary ethylenimine (BEI), photochemical inactivation, and combinations thereof. *See* Rueda et al. (2000) *Vaccine* 19:726-34 and Henzler & Kaiser (1998) *Nat Biotechnol* 16:1077-9. Preferably, inactivation does not significantly reduce viral antigenicity and/or activity. Viral inactivation is assayed using standard methods for determining viral infectivity.

[0141] Pasteurization is a simple approach if the viruses can withstand thermal treatment sufficient for inactivation. Preferably, the heating is performed for a minimally sufficient time period to minimize damage to viral proteins. Optionally, viral damage can be minimized by the use of stabilizers and sodium citrate, saccharose, and/or glycine.

[0142] Alternately, chemical inactivation, for example mild pepsin processing at low pH values or exposure to detergents, can be used to disrupt the lipid bilayer and thus is particularly useful for inactivating enveloped viruses, including baculovirus. *See* U.S. Patent Nos. 4,820,805 and 4,764,369. Aziridine binary ethylenimine is a potent alkylating agent that inactivates virus by selectively interacting with nucleophylic groups of nucleic acids but not proteins.

[0143] In one embodiment of the invention, viral inactivation is achieved via a photochemical reaction. According to this approach, a radiation sensitizing chemical compound is added to a liquid suspension of non-pathogenic viruses, and the mixture is exposed to UV light or ionizing (γ or X-ray) radiation.

[0144] Psoralen, and compounds with a linear tricyclic structure resembling psoralen, are capable of evoking photosensitization. Psoralens are bi-functional photoreactive molecules, which form covalent bonds with nucleic acids in the presence of long wavelength ultraviolet light. Psoralen molecules intercalate into DNA duplexes and

then photoreact to cross link the individual strands of the DNA. *See* Hwang et al. (1996) *Biochem Biophys Res Commun* 219:191-7. The crosslinking renders the DNA unable to replicate or to be transcribed. Commercially available psoralen compounds include 8-methoxypsoralen (methoxsalen) and 4,5', 8 trimethyl psoralen (trioxalen). The wavelengths most effective for photochemical inactivation using psoralen are in the range between 320 nm and 380 nm, with maximum effectiveness between 33 nm and 360 nm. *See* Pathak, M (1974) in Sunlight and Man, eds. Pathak, M & Fitzpatrick, T, University of Tokyo Press, Tokyo.

[0145] Additional photosensitizing agents include halogenated psoralens, angelicins, khellins and coumarins, which each contain a halogen substituent and a water solubilization moiety, such as, quaternary ammonium ion or phosphonium ion. It is believed that the substitution of halogen atoms, particularly bromine atoms, on psoralen molecules increases the binding constant of the sensitizer to DNA due to the hydrophobic nature of bromine. Brominated photosensitizing agents are also preferred in that only one photon of light is required to activate the brominated sensitizer, whereas two photons are required to effect DNA crosslinking using non-brominated psoralens. *See* U.S. Patent No. 5,418,130.

[0146] A representative method for photochemical inactivation is described in Example 10, which employs a combination of trioxalen and long wave length UV illumination. *See also* Weightman & Banks (1999) *J Virol Methods* 81:179-82 and Cotten et al. (1992) *Proc Natl Acad Sci USA* 89:6094-8.

[0147] To preserve antigenic characteristics of the virus, psoralen inactivation of live virus can be performed in a non-oxidizing atmosphere. By excluding oxygen and other oxidizing species from the inactivation medium, degradation of antigens via irradiation with ultraviolet light is largely prevented. *See* U.S. Patent No. 5,106,619. Similarly, antioxidants/quenchers can be used to minimize free radicals and other reactive oxygen species that are generated by exposure to short wave length UV light, and to thereby minimize protein damage. *See e.g.*, Marx et al. (1996) *Photochem Photobiol* 63:541-6.

[0148] In another embodiment of the invention, viral inactivation comprises modification of viral genes, whereby the virus is impaired or unable to replication. For example, a virus can be genetically modified to include one or more temperature-sensitive mutations in viral essential genes. The virus is produced and

grown in Sf9 or Tn5 cultures at the permissive temperature (*e.g.*, 25°C). When the virus is introduced into a mammal subject during treatment, the temperature is non-permissive (*e.g.*, 37°C) such that the temperature-sensitive genes would be poorly expressed, or the resultant gene products would have impaired function, and the virus would be crippled.

[0149] Representative temperature-sensitive mutations that could be generated include genes that are required for viral infection. For example, temperature-sensitive mutations in the gene encoding PKIP, a protein which interacts with a virus-encoded protein kinase, and in regulators of viral late gene transcription. At the non-permissive temperature, virus bearing such mutations show defects in viral infection. *See* McLachlin et al. (1998) *Virology* 246:379 and Partington et al. (1990) *Virology* 175:91.

[0150] Virus inactivation is assessed by a demonstrating a lost ability to replicate in a native host cell. Infectivity of a sample can be demonstrated using a standard plaque assay. When suitable methods to demonstrate infectivity of a particular virus are unknown, assessment of inactivation can rely on demonstrating inactivation of a model virus having similar biophysical and structural qualities. *See* Henzler & Kaiser (1998) *Nat Biotechnol* 16:1077-9. To render a virus completely inactive, the inactivation methods used in accordance with the present invention can include sequential exposure to an inactivating stimulus.

D.2. Carriers

[0151] To facilitate delivery of CCL21 and adjuvant to cancer cells in a subject, a composition that is administered to elicit an anti-cancer response in a subject comprises: (a) an effective amount of CCL21; (b) and effective amount of adjuvant; and (c) a pharmaceutically acceptable carrier. Where appropriate, two or more carriers can be used together.

[0152] Representative carriers for therapeutic administration include nanospheres (Manome et al., 1994; Saltzman and Fung, 1997), a glycosaminoglycan (*e.g.*, U.S. Patent No. 6,106,866), fatty acids (*e.g.*, U.S. Patent No. 5,994,392), fatty emulsions (*e.g.*, U.S. Patent No. 5,651,991), lipids and lipid derivatives (*e.g.*, U.S. Patent No. 5,786,387), collagen (*e.g.*, U.S. Patent No. 5,922,356), polysaccharides and derivatives thereof (*e.g.*, U.S. Patent No. 5,688,931), nanosuspensions (*e.g.*, U.S.

Patent No. 5,858,410), polymeric micelles or conjugates (*e.g.*, U.S. Patent Nos. 4,551,482, 5,714,166, 5,510,103, 5,490,840, and 5,855,900), and polysomes (*e.g.*, U.S. Patent No. 5,922,545).

[0153] For delivery of an adjuvant comprising a viral component, the carrier can further comprise a gene therapy vector, including a viral vector or a plasmid vector. Suitable viral vectors for gene expression include adenoviruses, adeno-associated viruses (AAVs), retroviruses, pseudotyped retroviruses, herpes viruses, vaccinia viruses, and Semiliki forest virus.

[0154] A carrier can be selected to effect sustained bioavailability of a non-pathogenic virus to a site in need of treatment. The term "sustained bioavailability" encompasses factors including but not limited to prolonged release of a non-pathogenic virus from a carrier, metabolic stability of a non-pathogenic virus, systemic transport of a composition comprising a non-pathogenic virus, and effective dose of a non-pathogenic virus.

[0155] Representative compositions for sustained bioavailability can include but are not limited to polymer matrices, including swelling and biodegradable polymer matrices, (U.S. Patent Nos. 6,335,035; 6,312,713; 6,296,842; 6,287,587; 6,267,981; 6,262,127; and 6,221,958), polymer-coated microparticles (U.S. Patent Nos. 6,120,787 and 6,090,925) a polyol:oil suspension (U.S. Patent No. 6,245,740), porous particles (U.S. Patent No. 6,238,705), latex/wax coated granules (U.S. Patent No. 6,238,704), chitosan microcapsules, and microsphere emulsions (U.S. Patent No. 6,190,700).

D.3. Formulation, Dose and Administration

[0156] Suitable formulations for administration of a composition of the invention to a subject include aqueous and non-aqueous sterile injection solutions which can contain anti-oxidants, buffers, bacteriostats, antibacterial and antifungal agents (*e.g.*, parabens, chlorobutanol, phenol, ascorbic acid, an thimerosal), solutes that render the formulation isotonic with the bodily fluids of the intended recipient (*e.g.*, sugars, salts, and polyalcohols), suspending agents and thickening agents. The formulations can be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and can be stored in a frozen or freeze-dried

(lyophilized) condition requiring only the addition of sterile liquid carrier immediately prior to use.

[0157] Compositions useful for injection into a host include sterile aqueous solutions or dispersions, and sterile powder for the preparation of sterile injectable solutions or dispersions. An injectable composition should be fluid to the extent that administration via a syringe is readily performed. Suitable solvents include water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol), and mixtures thereof. Fluidity can be maintained, for example, by the use of a coating such as lecithin and/or by minimization of particle size.

[0158] In accordance with the methods of the present invention, co-administration of CCL21 and an adjuvant can involve preparation of a therapeutic composition comprising both CCL21 and an adjuvant. Alternatively, co-administration can comprise administration of a CCL21 composition and a separate adjuvant composition to a same subject in need of treatment. The separate CCL21 and adjuvant compositions can be administered sequentially or simultaneously, as required to elicit the desired anti-cancer response.

[0159] A therapeutic composition of the present invention can be administered to a subject intratumorally, peritumorally, systemically, parenterally (*e.g.*, intravenous injection, intra-muscular injection, intra-arterial injection, and infusion techniques), orally, transdermally (topically), intranasally (inhalation), and intramucosally. A delivery method is selected based on considerations such as the type of the type of carrier or vector, therapeutic efficacy of the composition, and the condition to be treated.

[0160] In one embodiment of the present invention, CCL21 and/or an adjuvant are administered by direct injection into a tumor or into a peritumor site. The term "peritumor site" refers to a site less than about 15 cm from an outer edge of a tumor, more preferably less than about 10 cm from an outer edge of a tumor, still more preferably less than about 5 cm from an outer edge of a tumor, still more preferably less than about 1 cm from an outer edge of a tumor, and still more preferably less than about 0.1 cm from an outer edge of a tumor. A composition of the invention can be delivered to one or more tumor and/or peritumor sites. Preferably, a composition of the invention is administered at multiple sites within a tumor and/or surrounding a tumor.

[0161] In another embodiment of the invention, wherein the cancer is a non-neoplastic growth CCL21 and a non-pathogenic virus are administered at a lesional or perilesional site. The term "perilesional site" refers to a site less than about 15 cm from an outer edge of a non-neoplastic growth, more preferably less than about 10 cm from an outer edge of a non-neoplastic growth, still more preferably less than about 5 cm from an outer edge of a non-neoplastic growth, still more preferably less than about 1 cm from an outer edge of a non-neoplastic growth, and still more preferably less than about 0.1 cm from an outer edge of a non-neoplastic growth. A composition of the invention can be delivered to one or more lesional and/or perilesional sites. Preferably, a composition of the invention is administered at multiple sites within a non-neoplastic growth and/or surrounding a non-neoplastic growth.

[0162] The present invention provides that an effective amount of a therapeutic composition is administered to a subject. The term "effective amount" is used herein to describe an amount of CCL21 and an amount of adjuvant, when used in combination, are sufficient to elicit anti-tumor activity. As disclosed herein, representative anti-cancer activities include but are not limited to cancer cell cytolysis, inhibition of cancer growth, inhibition of cancer metastasis, and/or cancer resistance.

[0163] Actual dosage levels of active ingredients in a therapeutic composition of the invention can be varied so as to administer an amount of the composition that is effective to achieve the desired therapeutic response for a particular subject. Administration regimens can also be varied as required to elicit anti-cancer activity. A single injection or multiple injections can be used. The selected dosage level and regimen will depend upon a variety of factors including the activity of the therapeutic composition, formulation, the route of administration, combination with other drugs or treatments, the disease or disorder to be treated, and the physical condition and prior medical history of the subject being treated. Determination and adjustment of an effective amount or dose, as well as evaluation of when and how to make such adjustments, are known to those of ordinary skill in the art of medicine.

[0164] For additional guidance regarding formulation, dose and administration regimen, *see* Berkow et al. (1997) The Merck Manual of Medical Information, Home ed. Merck Research Laboratories, Whitehouse Station, New Jersey; Goodman et al.

(1996) Goodman & Gilman's the Pharmacological Basis of Therapeutics, 9th ed. McGraw-Hill Health Professions Division, New York; Ebadi (1998) CRC Desk Reference of Clinical Pharmacology. CRC Press, Boca Raton, Florida; Katzung (2001) Basic & Clinical Pharmacology, 8th ed. Lange Medical Books/McGraw-Hill Medical Pub. Division, New York; Remington et al. (1975) Remington's Pharmaceutical Sciences, 15th ed. Mack Pub. Co., Easton, Pennsylvania; Speight et al. (1997) Avery's Drug Treatment: A Guide to the Properties, Choice, Therapeutic Use and Economic Value of Drugs in Disease Management, 4th ed. Adis International, Auckland / Philadelphia, Pennsylvania.

[0165] It will be readily apparent to one skilled in the art that following a review of the present disclosure, the therapeutic methods for administration of a non-pathogenic virus can be variably performed to elicit an anti-cancer response.

[0166] In accordance with long-standing convention, the terms "a," "an," and "the" are used to refer to one or more. The term "about", as used herein when referring to a measurable value, for example a peritumoral or perilesional distance, is meant to encompass variations of ±20% or ±10%, more preferably ±5%, even more preferably ±1%, and still more preferably ±0.1% from the specified amount, as such variations are appropriate to perform a disclosed method or otherwise carry out the present invention.

Examples

[0167] Example 1. Preparation of Recombinant Baculovirus

[0168] The full-length sequence encoding human CCL21 (GenBank Accession No. NM 002989) was cloned into the baculovirus transfer vector pVL1392 (Pharmingen of San Diego, California) and co-transfected with BACULOGOLD® WT genomic DNA (Pharmingen of San Diego, California) using methods recommended by the vendor. The recombinant baculovirus obtained from this procedure was subcloned by plaque-purification on Sf9 insect cells to yield several isolates expressing human CCL21. A clone was selected for its exceptional expression characteristics compared to the original virus. Amplification of this baculovirus isolate was performed at low multiplicity of infection (MOI) to generate high-titer, low passage stock for protein production. The baculovirus expressing human CCL21 was

designated BV422. Additional recombinant baculovirus were similarly prepared. For example, baculovirus expressing intracellular Raf protein was prepared and designated BV762.

[0169] Protein production and budded baculovirus production involved infection of suspended *Trichoplusia ni* (Tn5) cells in protein-free media at multiplicity of infection (MOI) of 2-10 for 48 hours. BV422 culture supernatants, which included the recombinantly expressed CCL21, were collected by centrifugation, clarified by filtration and prepared for column purification.

[0170] Example 2. Tumor Growth Inhibition in an Animal Model of Lung Cancer

[0171] C57BL/6 mice at 9-11 weeks of age were allowed to acclimate for a minimum of 7 days prior to inoculation with tumor cells. Mice were inoculated s.c. at the right flank with 2 X 10^5 early passage (<10 passages) 3LL-HM tumor cells. Tumor size was measured twice per week. When tumors reached 50-100 mm^3 (typically 7 days after tumor inoculation), mice were randomized into groups. Baculovirus-expressed CCL21 was administered intratumorally to tumor-bearing mice. Dose and administration regimen were varied to optimize tumor inhibition. When tumor volume in any group reached 3000 mm^3 (typically 33-35 days after inoculation in mice of the control group), mice were sacrificed.

[0172] As shown in Figure 1, intratumoral administration of baculovirus-expressed CCL21 resulted in growth delay of 3LL tumors. CCL21 dose was optimized to achieve complete inhibition of tumor growth. An administration regimen that included 2 or 3 injections at a relatively higher dose showed similar efficacy when compared to an administration regimen that included 6 injections at a relatively lower dose. Some tumor inhibition was also seen using a single dose.

[0173] Example 3. Tumor Growth Inhibition in an Animal Model of Breast Cancer

[0174] Balb/c mice at 9-11 weeks of age were allowed to acclimate for a minimum of 7 days prior to inoculation with tumor cells. Mice were inoculated s.c. at the right flank with 2 X 10^5 4T1 cells. Tumor size was measured twice per week. When tumors reached 50-100 mm^3 (typically 7 days after tumor inoculation), mice were randomized into groups. Baculovirus was administered intratumorally to tumor-bearing mice. Dose was varied to determine an optimal effective dose. When tumor

volume in any group reached 3000 mm^3 (typically 33-35 days after inoculation in mice of the control group), the mice were sacrificed. As shown in Figure 2, intratumoral administration of CCL21 resulted in growth delay of 4T1 tumors.

[0175] Example 4. Tumor Growth Inhibition in a Melanoma Model

[0176] The mouse melanoma cell line, B16-BL6, is used to establish subcutaneous tumors in 6-8 week old pink-skinned female BDF-1 mice (Charles River Laboratories of Boston, Massachusetts). To produce cutaneous tumors, 10^6 B16-BL6 cells in 0.2 ml media are injected into the upper back region of 6-8 week old female BDF-1 mice on day 0. Cell viability is assessed using trypan blue exclusion before and after cell injection. The number of dead cells before injection is typically not more than 10% of the total number of cells. By day 6, tumors are typically 5-10 mm in diameter.

[0177] Baculovirus-expressed CCL21 are prepared as described in Example 1. Baculovirus are administered subcutaneously, at a site approximately 3 mm away from each tumor, on days 3 and 4. Tumor volume is measured daily for three weeks. Mice are sacrificed when tumor volume reaches 4000 mm^3.

[0178] Example 5. Resistance to Tumor Re-challenge

[0179] Mice bearing tumors were prepared and treated with baculovirus as described in Example 3. Tumors were surgically removed 2 days after the final dose of baculovirus and subjected to tumor re-challenge. Mice were anesthetized using 200 µl ketamine/xylazine mixture (4:1 ketamine:xylazine diluted 10-fold in phosphate-buffered saline) injected intraperitoneally, the tumor was resected, and the wound was closed with staples. One to four days following tumor resection, mice were re-challenged by subcutaneous administration of 2 X 10^5 4T1 cells at a site other than the original tumor site. Re-challenge tumor volume was measured twice per week. Figure 4 shows that mice treated with baculovirus-expressed CCL21 were able to resist re-challenge with 4T1 tumor cells.

[0180] Example 6. Inhibition of Tumor Metastases

[0181] Mice bearing tumors were prepared and treated with baculovirus as described in Example 3. Mice were sacrificed when the control group showed signs of severe sickness due to lung metastases. Typical indicators include laborious breathing, greasy fur, and weight loss. The lungs were harvested and preserved in Buoin's

solution. The presence of lung metastases was determined. Figure 3 shows that baculovirus-expressed CCL21 significantly inhibited tumor metastasis.

[0182] Example 7. Baculovirus is an Active Component of Baculovirus-Expressed CCL21

[0183] The anti-tumor activity of baculovirus-expressed hCCL21, as described in Examples 2-3 and 5-6, requires the adjuvant activity of baculovirus. The experimental results disclosed herein contradict the independent findings of Sharma et al. and Arenberg et al. *See* Sharma et al. (2000) *J Immunol* 154:4558-63 and Arenberg et al. (2001) *Cancer Immunol Immunother* 49:587-92. These prior studies attributed anti-tumor responses to the activity of CCL21, but did not teach nor suggest the necessity of an adjuvant. Following a review of these references, one skilled in the art would not have been motivated to perform methods for cancer therapy that minimally employ CCL21 and an adjuvant. Rather, only CCL21 was identified as a necessary component for the anti-tumor responses described therein. In contrast to the Sharma et al. and Arenberg et al. references, the present invention teaches that anti-tumor responses, which were previously interpreted to be the result of CCL21, require a combination of CCL21 and adjuvant.

[0184] To clarify the source of anti-tumor activity observed in some preparations of baculovirus-expressed CCL21, such preparations were filtered using a 0.2 μm filter to remove high molecular weight substances (*i.e.*, those substance having a molecular weight greater than 50 kDa) from the preparation. As shown in Figure 6, filtered preparations of baculovirus-expressed CCL21 were insufficient to effect tumor remission in a 3LL tumor model. The concentration of CCL21 in the any given sample was unchanged by filtration. In addition, filtered preparations were sufficient for CCL21-induced chemotaxis *in vitro*. These results suggested that a high molecular weight contaminating substance in baculovirus-expressed CCL21 preparations was required for robust anti-tumor activity, but not for CCL21-induced chemotaxis *in vitro*.

[0185] In contrast to the variability observed in vivo, all CCL21 preparations were sufficient to induce chemotaxis of lymphocytes *in vitro*. *See* Table 1 and Figure 7. These results suggested that a high molecular weight contaminating substance in baculovirus-expressed CCL21 was required for robust anti-tumor activity, but not for CCL21-induced chemotaxis *in vitro*.

[0186] Table 1. Activity of Recombinant CCL21 Preparations

CCL21 Species of Origin	Host for Heterologous Expression	Lot#	*In Vitro* chemotaxis activity	*In Vivo* anti-tumor activity
Mouse	Baculovirus	MBAY1	+	+
Mouse	Baculovirus	MBMC1	+	+
Mouse	Baculovirus	MBDS1	+	-
Mouse	Baculovirus	MBDS2	+	+
Human	Baculovirus	HBDS1	+	-
Human	Baculovirus	HBMC1	+	-
Human	Baculovirus	HBDS2	+	+
Human	Baculovirus	HBDS3	+	+
Human	Baculovirus	HBPG1	+	+
Human	Baculovirus	HBDS4	+	+
Mouse	*E. coli*	PeproTech	+	-
Human	*E. coli*	HEDS1	+	-
Human	*E. coli*	HEDS4	+	-
Human	*E. coli*	HEPG3	+	-
Human	*E. coli*	PeProTech[1]	+	-
Human	Chinese Hamster Ovary (CHO) Cells	HCDS1	+	-
Human	Yeast	HYPG4	+	-

[1]Available from PreproTech EC Ltd. of Rocky Hill, New Jersey

[0187] Baculovirus was found to be a contaminant of baculovirus-expressed CCL21 prepared as described in Example 1. Baculovirus-expressed CCL21 was used to prepare a Western Blot, which was probed with an antibody that specifically recognizes the baculoviral protein gp64. As shown in Figure 8, gp64 was detected in baculovirus-expressed CCL21 preparations. In addition, lots of baculovirus-expressed CCL21 that showed anti-tumor activity had relatively high titers of live virus, while inactive lots had relatively lower titers of live virus (Table 2).

[0188] Table 2. Viral Titer Correlates with Anti-Tumor Activity of Baculovirus-Expressed CCL21 Preparations

Lot	Concentration (mg/ml)	PFU/ml	Regimen (µg/dose)	PFU/dose	Total PFU delivered	Activity
HBDS4 (filtered)	0.5	<20	25	<1	<6	-
HBDS4[1]	0.5	<30	25	<1.5	<9	-
HBDS4[2]	0	600	25	30	180	-
HBMC1	0.5	1.0×10^4	100	5.0×10^2	3.0×10^3	-
HBDS1	1.8	1.0×10^6	25	1.4×10^4	8.3×10^4	+/-
HEPG3 +bv[3]	0.5	1.3×10^6	25	6.5×10^4	3.9×10^4	+
HBDS4	0.8	5.0×10^5	25	1.6×10^4	9.00×10^4	+
HBPG1	1.8	2.7×10^7	25	3.8×10^5	2.00×10^6	+

[1]small fraction; [2]large fraction; [3]bv, baculovirus

[0189] Figure 9 shows that the anti-tumor activity of CCL21 preparations is substantially abolished upon filtering of the preparation to remove high molecular weight contaminants, including baculovirus.

[0190] Example 8. Baculovirus Activates Dendritic Cell Maturation

[0191] Addition of wild type baculovirus to dendritic cell (DC) cultures induced their maturation, as evidenced by increased cell surface expression of activation markers. As shown in Figures 10A and 10B, baculovirus activates mouse bone marrow-derived DCs and human monocyte-derived DCs.

[0192] Mouse DCs were prepared from bone marrow according to standard methods. Briefly, bone marrow was isolated from female Balb/c or C57Bl/6 mice, 6-8 weeks old, (Charles River Laboratories of Holister, California) and frozen (-80°C) in heat-inactivated fetal bovine serum supplemented with 10% cell-culture grade

dimethyl sulfoxide (DMSO) at a density of 2 X 10^7 cells/ml. Frozen cell aliquots were rapidly thawed and washed to remove DMSO. Cells were plated in 150 mm suspension culture dishes containing 20 ml supplemented RPMI media (Sigma-Aldrich of St. Louis, Missouri) containing 200 U/ml murine GM-CSF (PreproTech of Rocky Hill, New Jersey). On day 3 of culture, cells were again supplemented with murine GM-CSF, and on day 5, one-half of the culture volume was centrifuged to replace fresh medium containing GM-CSF. BMDC were harvested by gentle pipetting.

[0193] Baculovirus and other control materials were added to the media on day 6. Cells were incubated an additional 18 hours prior to analysis of cells or supernatants. BMDC were analyzed for cell surface markers by FACS and were characterized as immature on day 6 prior to addition of stimuli by detection of markers for DC immaturity, including CD11c, CD11b, H-2K^d, I-A$^{d(low)}$, CD80$^{(low)}$, and CD86$^{(low)}$. Following overnight incubation with various stimuli, cells were washed and double-stained using anti-CD11c and anti-CD86 or anti-I-A antibodies and then analyzed by flow cytometry. Cells were gated on the live CD11c$^+$ population. Stimulation is expressed as the mean fluorescence intensity (MFI) divided by MFI from stained cells treated only with GM-CSF. Figure 10A shows that the expression of the DC activation marker CD86 and MHC class II (detected using anti-I-A antibodies) was increased in response to baculovirus. The levels of CD80 and CD40 were similarly elevated in response to baculovirus.

[0194] Human DC were derived from peripheral blood monocytes purified from the buffy coats of healthy volunteers by using anti-CD14 antibody-coated magnetic beads (Miltenyi Biotec of Auburn, California). Immature DC were harvested after 3-4 days of culture with interleukin-4 and GM-CSF (each 1000 U/ml; available from PreproTech of Rocky Hill, New Jersey). Cultures were routinely >90% CD1a positive by FACS (Pharmingen of San Diego, California). FACS analysis of DC activation markers was assessed by gating on live CD1a+ cells. Figure 10B shows that baculovirus induced elevated levels of CD86 and HLA-DR++.

[0195] Example 9. Baculovirus Activates CTLs *In Vivo*

[0196] Immunization of mice with baculovirus and a soluble protein antigen (HIV p24) induced a robust, antigen-specific CTL response. Spleens from immunized mice were harvested 2 weeks following the third immunization. Individual spleens were

combined such that 5 spleens were included in each sample. Spleen cells from immunized mice were cultured in a 24-well dish at 5 X 10^6 cells per well. Of these cells, 1 x 10^6 cells were sensitized with: (1) a synthetic p7g peptide, which is an H-2K^d restricted CTL epitope corresponding to amino acids 199-208 of HIV-1$_{SF2}$p24gag; and (2) a pGag^b peptide, which is an H-2D^b restricted CTL epitope corresponding to amino acids 390-398 of HIV-1$_{SF2}$p55gag. The peptides were used at a concentration of 10μM for 1 hour at 37°C. Splenocytes were then washed and co-cultured with the remaining 4 X 10^6 untreated cells. The splenocytes were stimulated as a bulk culture in 2 ml of splenocyte culture medium: RPMI 1640 (Sigma-Aldrich of St. Louis, Missouri) with 100 mM L-glutamine (Gibco of Grand Island, New York) and α-Mem (Minimum Essential medium Alpha Medium with L-glutamine, deoxyribonucleosides or ribonucleosides) (1:1), supplemented with 10% heat inactivated fetal calf serum (Hyclone of Logan, Utah), inactivated in a 56°C water bath for 30 minutes in 100 U/ml penicillin, 10 μg/ml streptomycin, 10 ml/L of 100mM sodium pyruvate and 50μM 2-mercaptoethanol. In addition, 5% Rat T-Stim IL2 (Rat T-Stim: Collaborative Biomedical Products of Bedford, Massachusetts) was used as a source of IL2 and was added to the culture media just before the cells were cultured.

[0197]After a stimulation period of 6-7 days, splenocytes were collected and used as effectors in a chromium release assay. Approximately 1 X 10^6 SV/Balb or MC57 target cells were incubated in 200 μl of medium containing 50 μCi of ^{51}Cr and with the correct peptide p7g, or a mismatched cell-target pair as the negative control, at a concentration of 1μM for 60 minutes and washed. Effector cells were cultured with 5 x 10^3 target cells at various effector to target ratios in 200 μl of culture medium in 96-well round or V-bottom tissue culture plates for 4 hours. The average counts per minute from duplicate wells was used to calculate percent specific release. Figure 11 shows that baculovirus was a potent adjuvant to induce cytolytic T cell responses.

[0198]Example 10. Photochemical Inactivation of Baculovirus

[0199]Two liter suspension cultures of *Trichoplusia ni* (Tn) cells are infected with baculovirus. Following incubation for 3 days at 28°C the infected cell suspension is harvested and clarified by centrifugation at 800 X *g* for 10 minutes. The titer of baculovirus in the harvested medium was determined by plaque assay in

Spodoptera frugiperda (Sf) cells, for example as described by King & Possee (1992) The Baculovirus Expression System: A Laboratory Manual, Chapman & Hall, London.

[0200] A stock solution of trioxalen is prepared at a concentration of 0.2 mg/ml in dimethyl sulfoxide (DMSO). Trioxalen is added to the infected cell suspension at a concentration of about 5-10 µg/ml and dispersed within the cell suspension by gentle shaking. The cell suspension is then poured into a seamless, stainless steel tray (*e.g.*, about 1 cm in depth) and placed on a rotating platform. A long wavelength (365 nm, 6W) UV lamp is placed directly above the tray at a distance of 1 cm from the liquid surface. Exposure to UV illumination is allowed to proceed for about 15 minutes, or for a period sufficient for virus inactivation.

[0201] To assess virus inactivation, the trioxalen/UV inactivated samples are titrated on insect cells. For example, aliquots are taken from the cell suspension, serially diluted, and used to inoculate Sf9 cell cultures. The medium is changed at about 16 hours post inoculation to minimize DMSO-induced cytotoxicity. The cultures are examined microscopically to assess cellular pathology 7 days post inoculation and, if negative, are passaged twice more to confirm virus inactivation. The cultures are also examined to identify cellular cytotoxicity.

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The references listed below as well as all references cited in the specification are incorporated herein by reference to the extent that they supplement, explain, provide a background for or teach methodology, techniques and/or compositions employed herein.

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It will be understood that various details of the invention can be changed without departing from the scope of the invention. Furthermore, the foregoing description is for the purpose of illustration only, and not for the purpose of limitation--the invention being defined by the claims appended hereto.

Abstract

A method for the treatment or prevention of cancer in a subject via administration of CCL21 in combination with a non-pathogenic virus. The cancer treatment or prevention can include inhibition of tumor growth, inhibition of tumor metastases, and/or resistance to tumor re-challenge.

FIG. 1

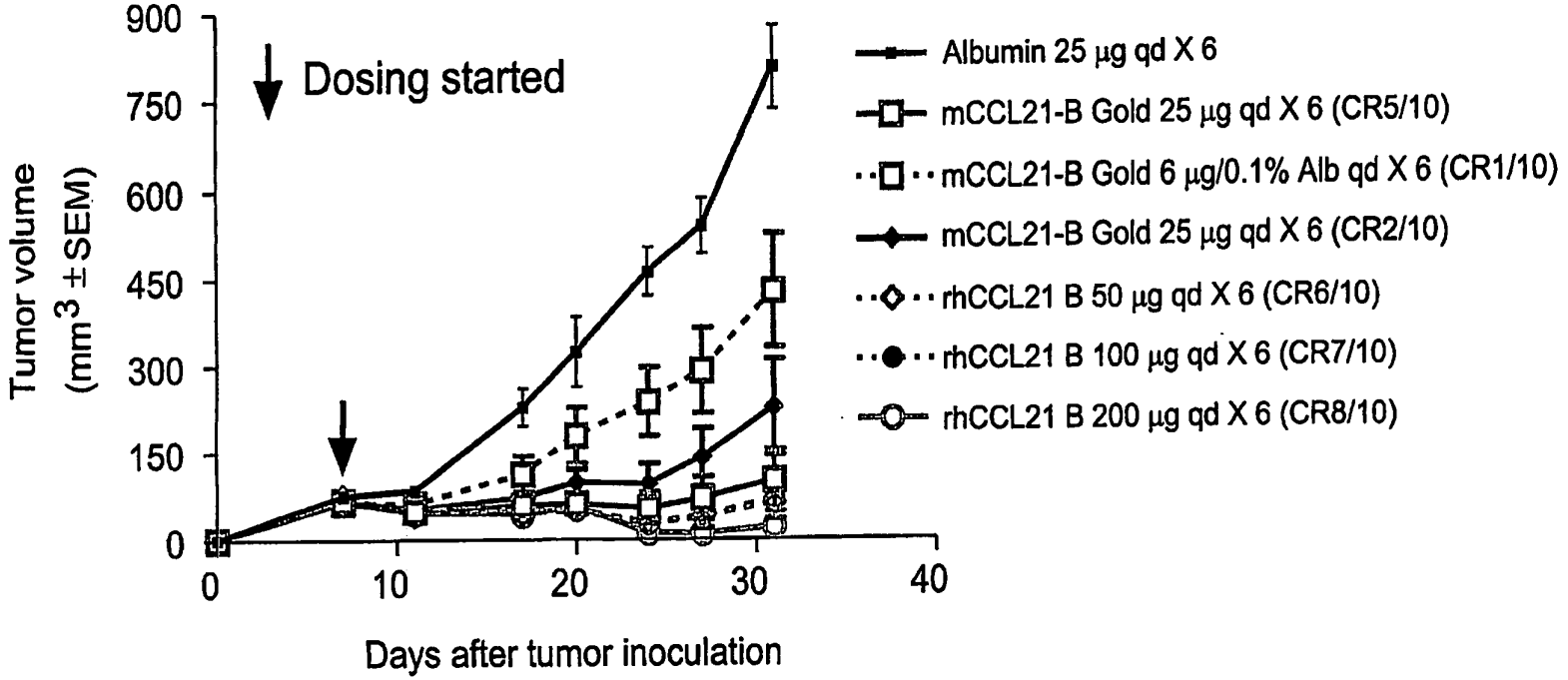
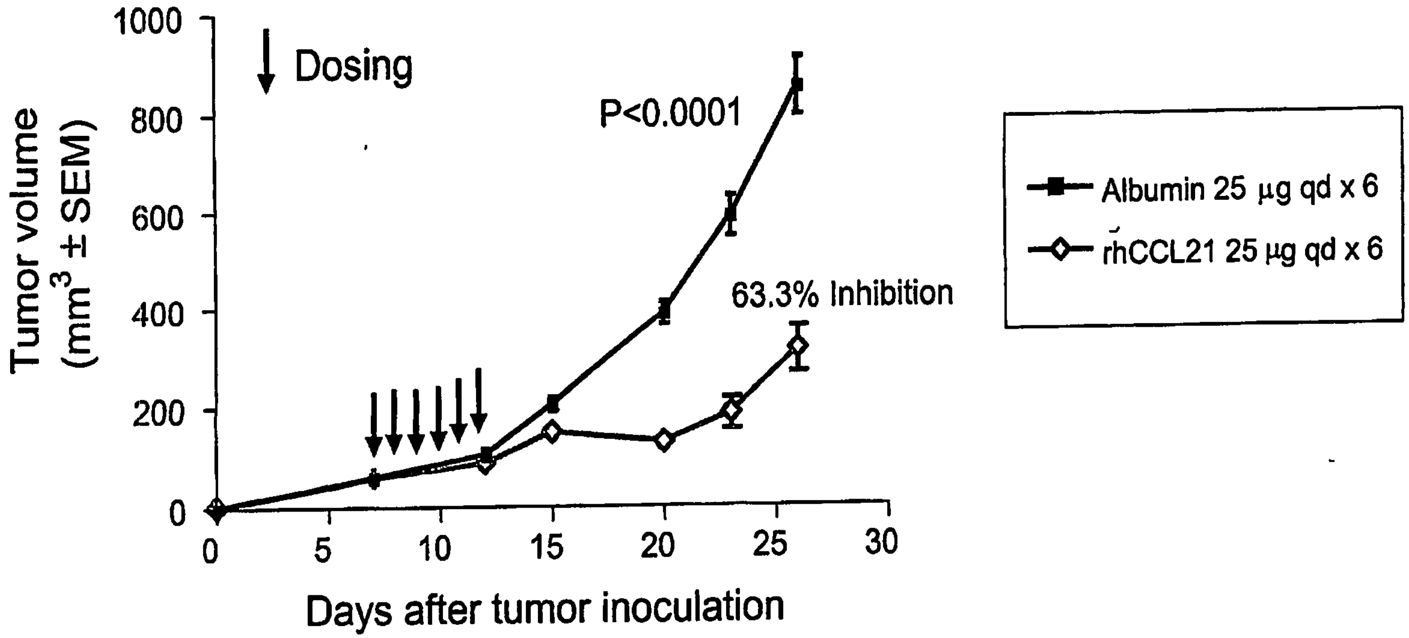


FIG. 2



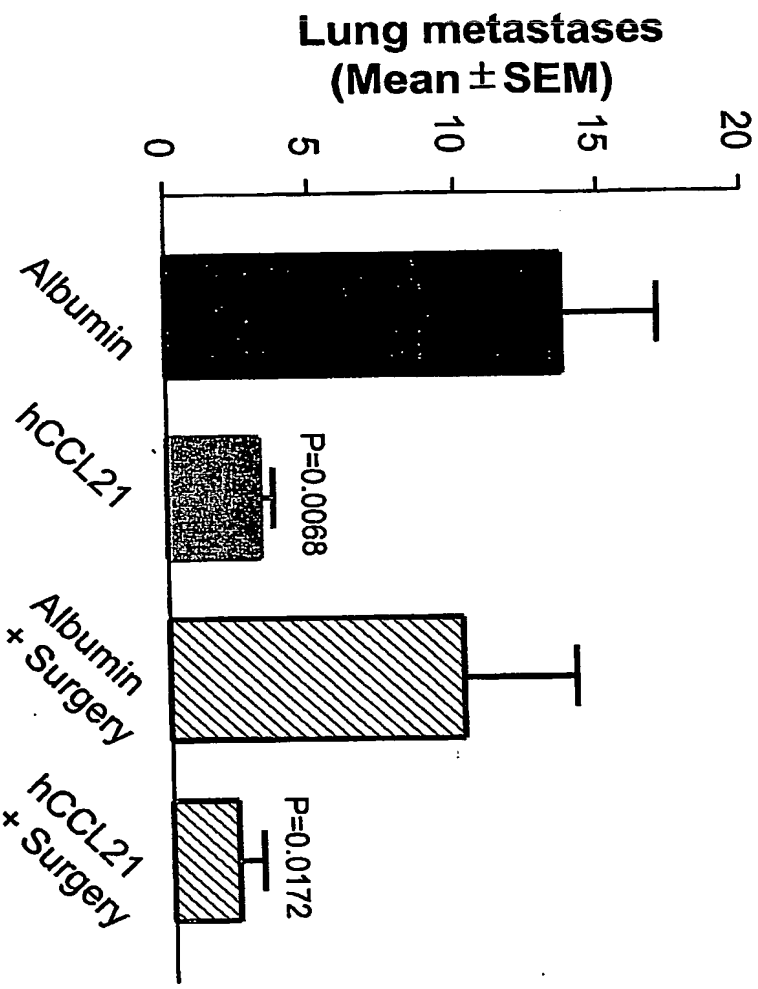

Lung metastases
(Mean ± SEM)
0
5
10
15
20
Albumin
hCCL21
P=0.0068
Albumin
+ Surgery
hCCL21
+ Surgery
P=0.0172

FIG. 3

FIG. 4

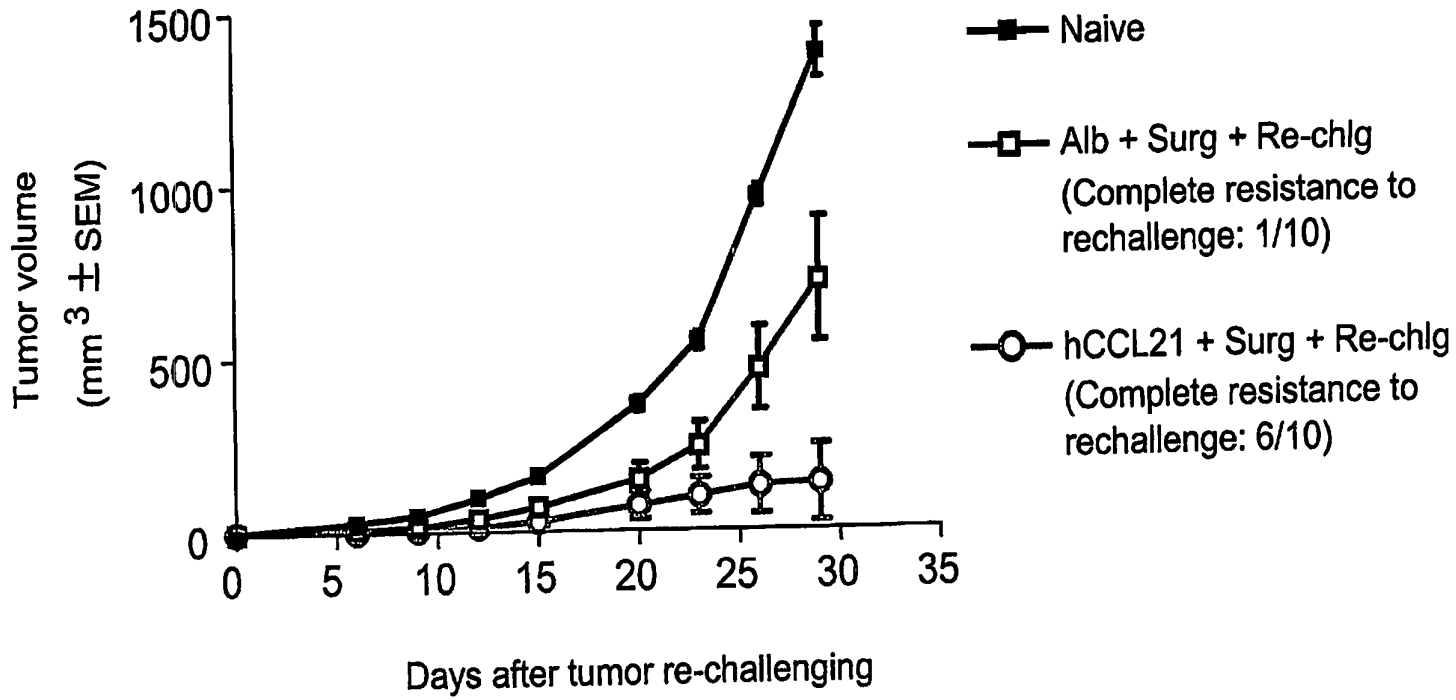


FIG. 5A

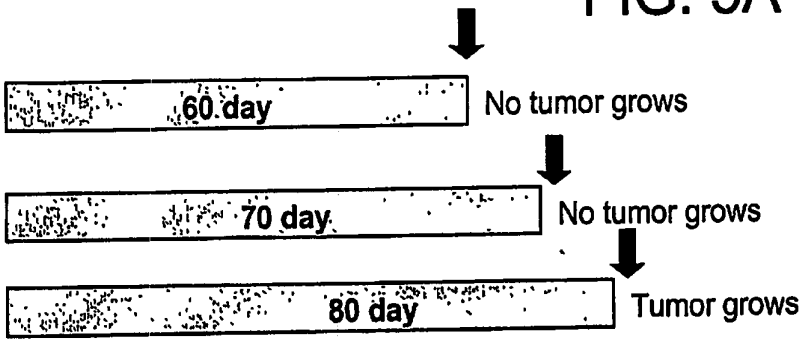


FIG. 5B

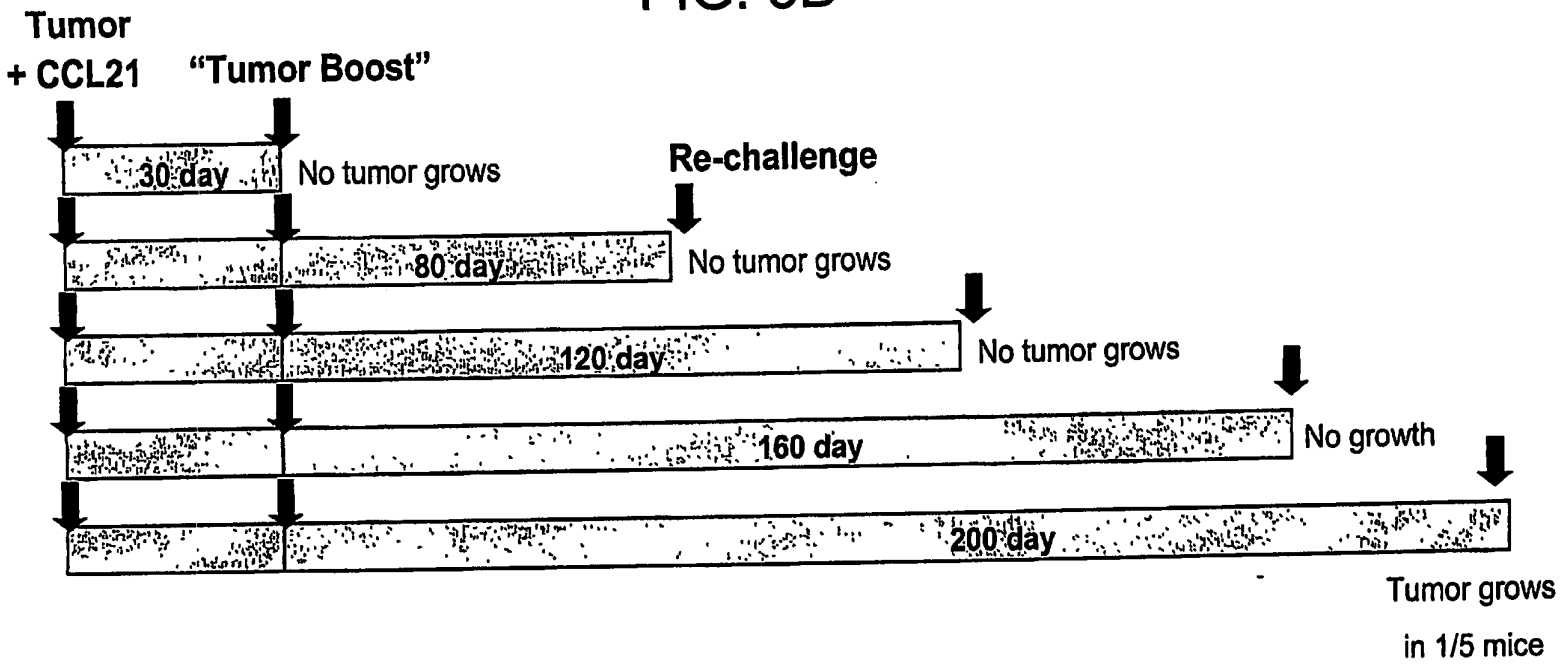


FIG. 6

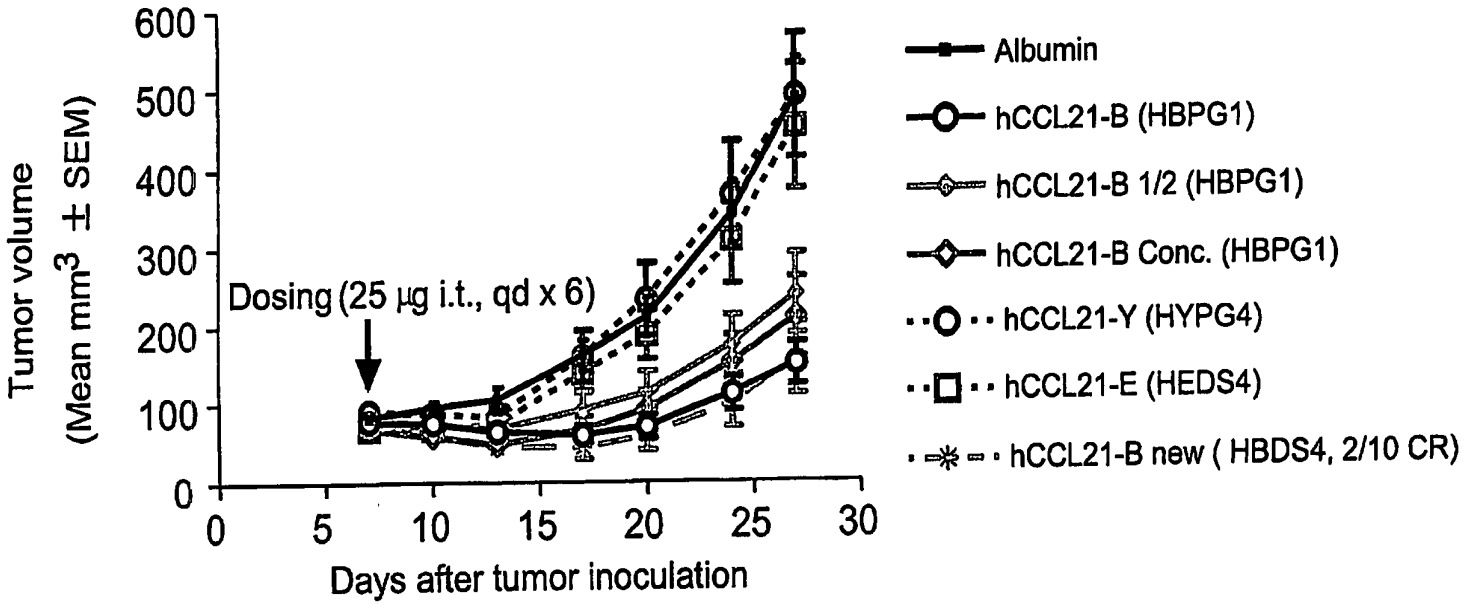


FIG. 7

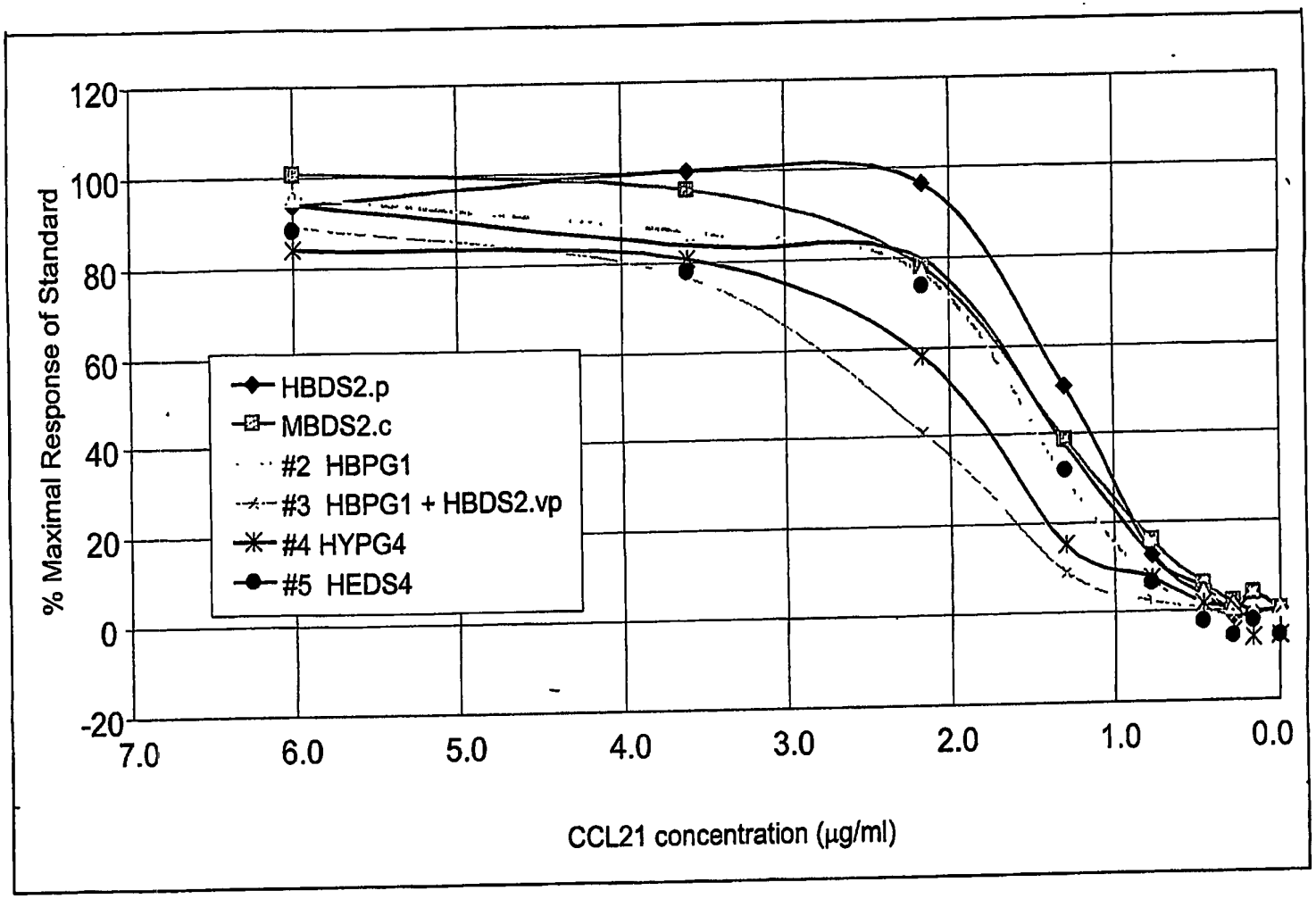
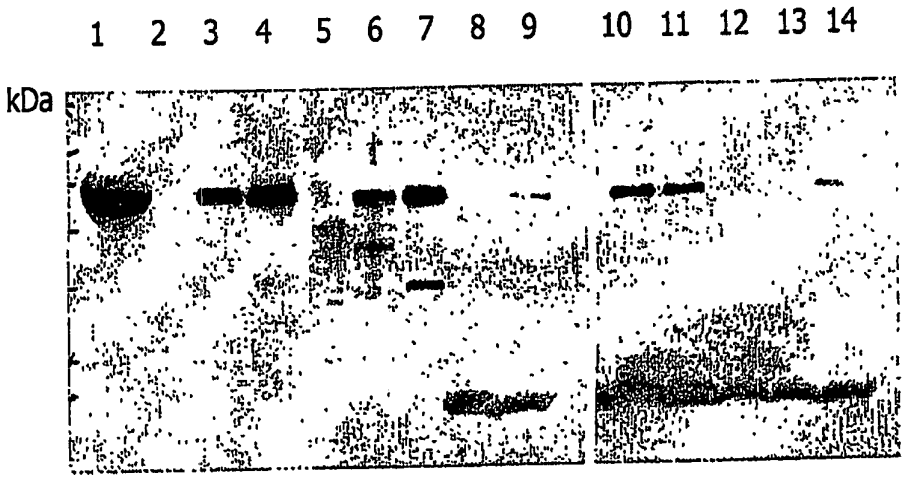
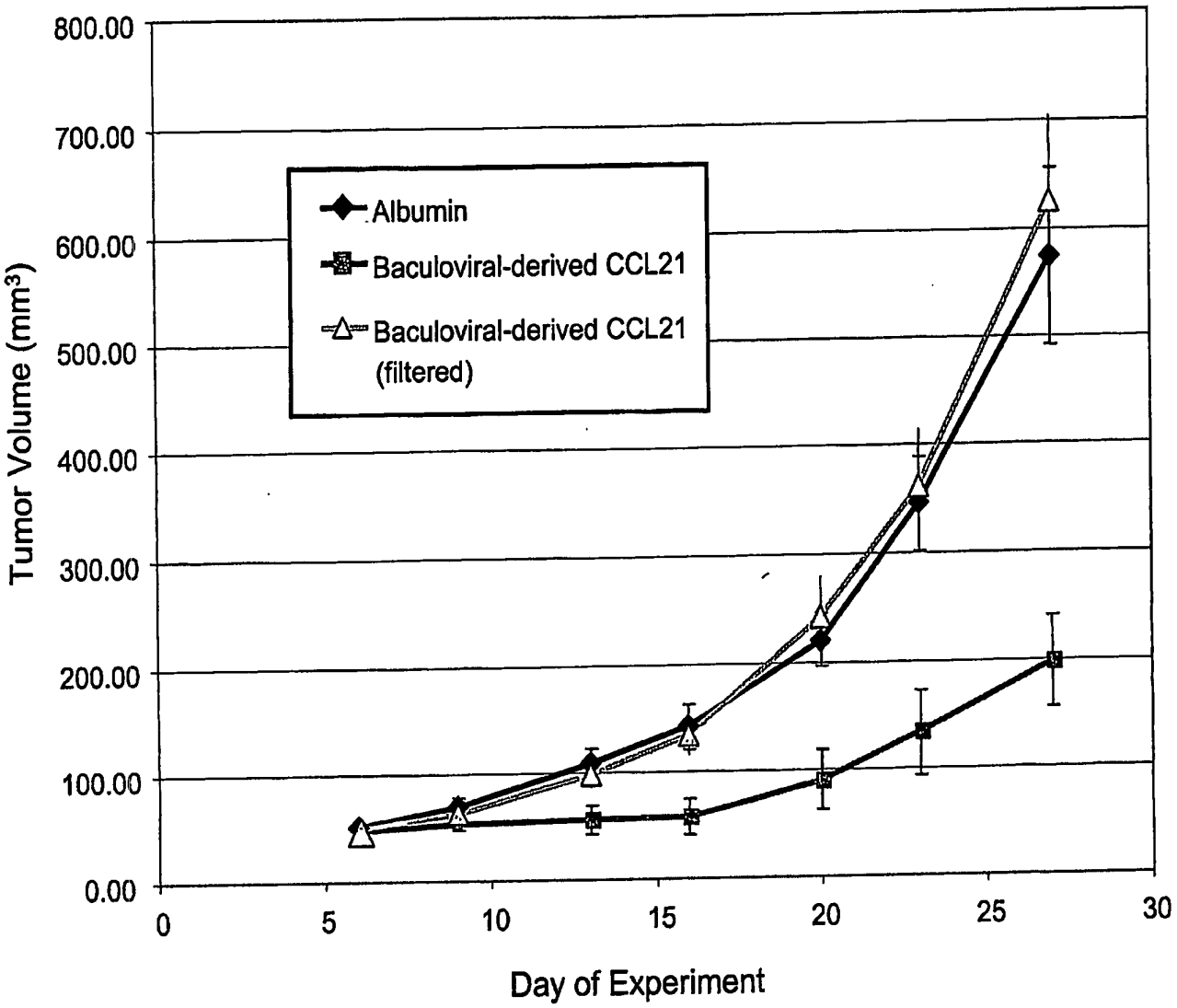

120
100
80
60
40
20
0
-20
% Maximal Response of Standard
HBDS2.p
MBDS2.c
#2 HBPG1
#3 HBPG1 + HBDS2.vp
#4 HYPG4
#5 HEDS4
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
CCL21 concentration (µg/ml)

FIG. 8



1- Clontech + baculovirus
2- uninfected Tn5 cells, 1x supernatant
3- mock infected cells, 1x supernatant
4- BV422 infected cells, 1x supernatant
5- uninfected cells
6- mock infected cells
7- BV422 infected cells
8- <50kD 5µg
9- >50kD 5µg
10->50kD 10µg
11- PG1 5µg
12- DS4 5µg
13- MC1 5µg
14- DS1 5µg

FIG. 9


800.00
700.00
600.00
500.00
400.00
300.00
200.00
100.00
0.00
Tumor Volume (mm³)
0
5
10
15
20
25
30
Day of Experiment
Albumin
Baculoviral-derived CCL21
Baculoviral-derived CCL21 (filtered)

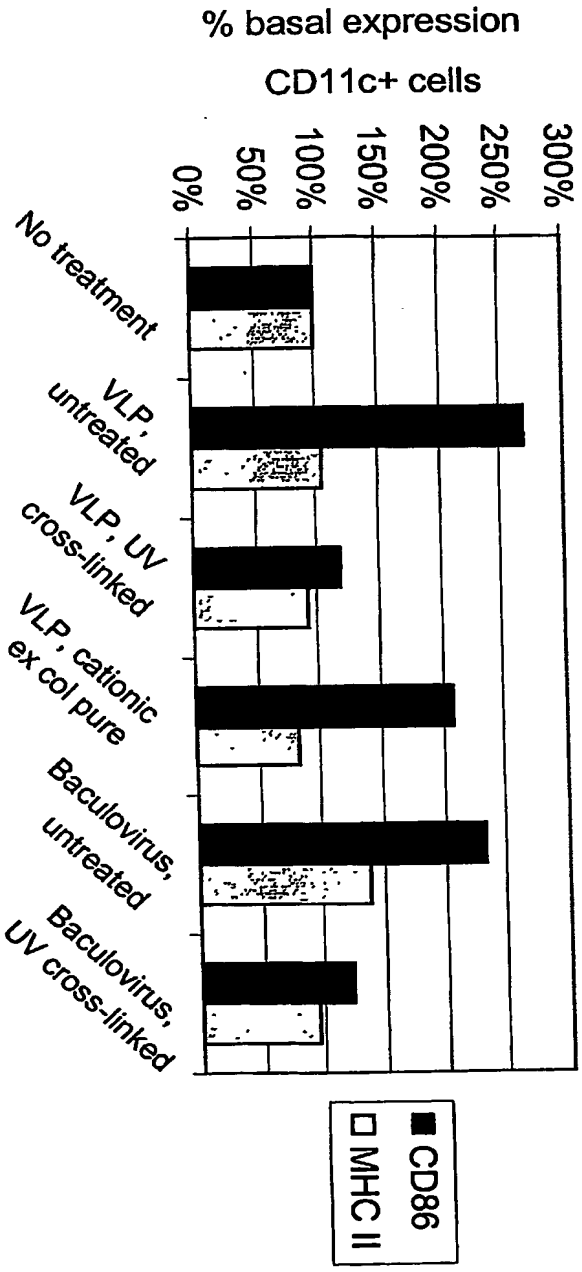

% basal expression
CD11c+ cells
0%
50%
100%
150%
200%
250%
300%
No treatment
VLP, untreated
VLP, UV cross-linked
VLP, cationic ex col pure
Baculovirus, untreated
Baculovirus, UV cross-linked
CD86
MHC II

FIG. 10A

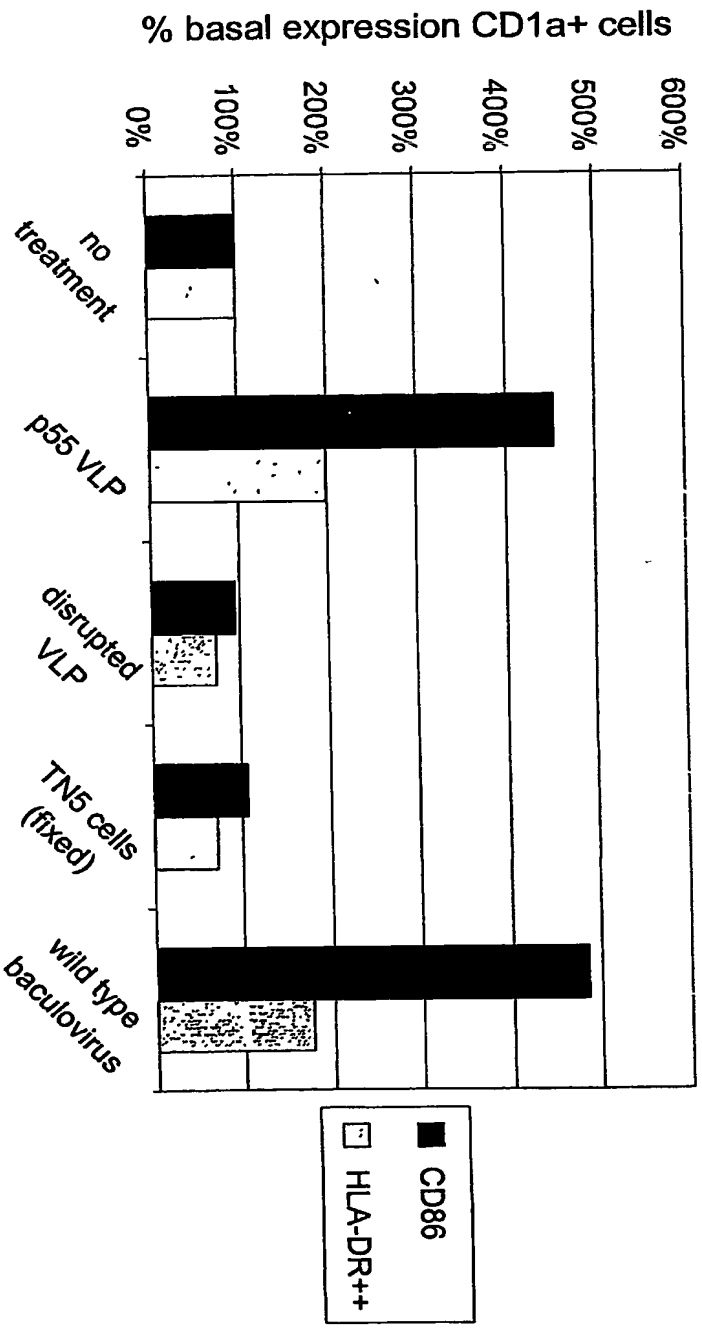
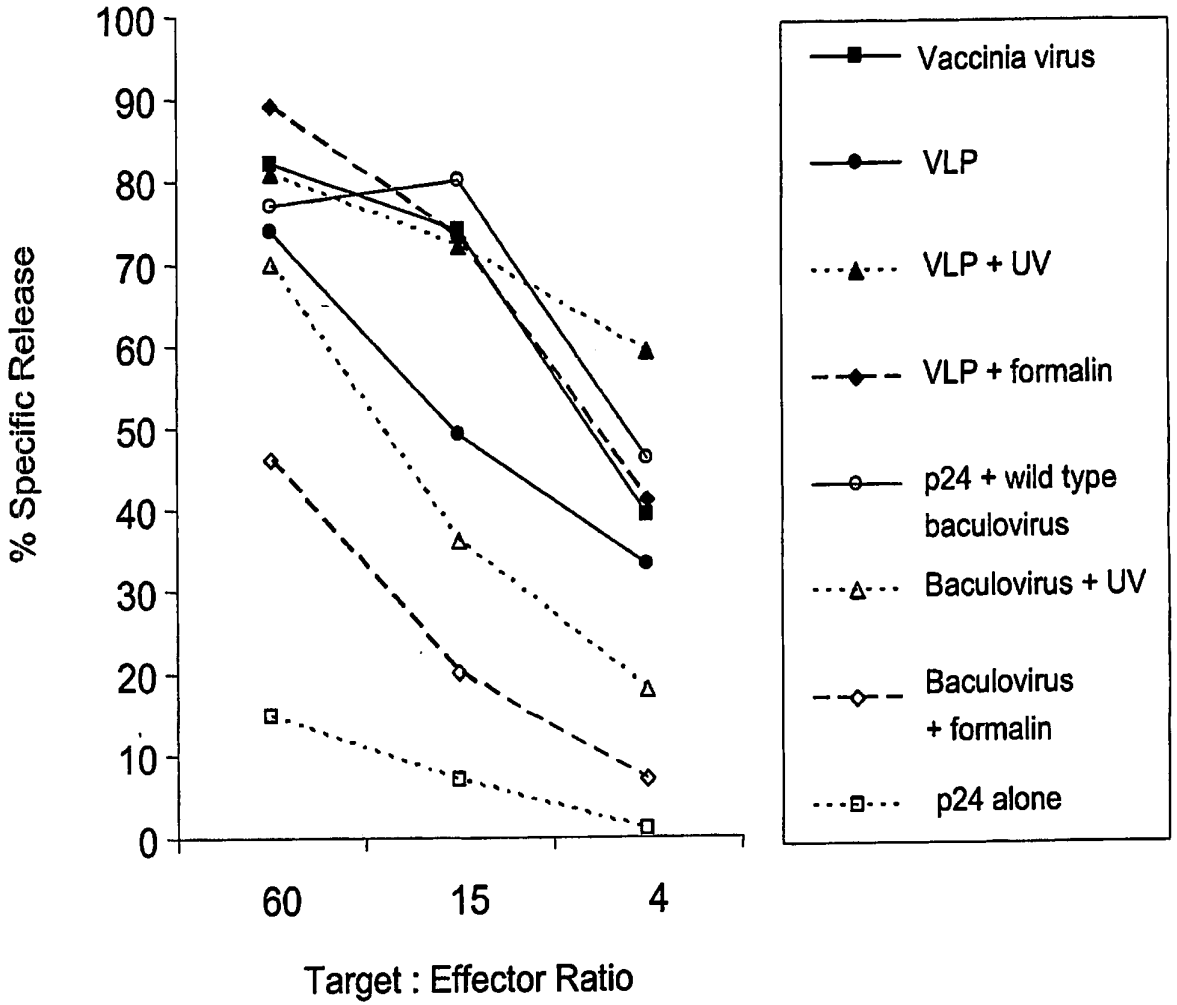

% basal expression CD1a+ cells
0%
100%
200%
300%
400%
500%
600%
no treatment
p55 VLP
disrupted VLP
TN5 cells (fixed)
wild type baculovirus
CD86
HLA-DR++

FIG. 10B

FIG. 11


100
90
80
70
60
50
40
30
20
10
0
% Specific Release
60
15
4
Target : Effector Ratio
Vaccinia virus
VLP
VLP + UV
VLP + formalin
p24 + wild type baculovirus
Baculovirus + UV
Baculovirus + formalin
p24 alone

Sequence Listing

<110> Chiron Corporation
Kavanaugh, Michael

<120> CCL21, IN COMBINATION WITH BACULOVIRUS, PROMOTES CANCER RESISTANCE

<130> 014024-0291804

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<170> PatentIn version 3.1

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Met Ala Gln Ser Leu Ala Leu Ser Leu
1 5

ctt atc ctg gtt ctg gcc ttt ggc atc ccc agg acc caa ggc agt gat 159
Leu Ile Leu Val Leu Ala Phe Gly Ile Pro Arg Thr Gln Gly Ser Asp
10 15 20 25

gga ggg gct cag gac tgt tgc ctc aag tac agc caa agg aag att ccc 207
Gly Gly Ala Gln Asp Cys Cys Leu Lys Tyr Ser Gln Arg Lys Ile Pro
30 35 40

gcc aag gtt gtc cgc agc tac cgg aag cag gaa cca agc tta ggc tgc 255
Ala Lys Val Val Arg Ser Tyr Arg Lys Gln Glu Pro Ser Leu Gly Cys
45 50 55

tcc atc cca gct atc ctg ttc ttg ccc cgc aag cgc tct cag gca gag 303
Ser Ile Pro Ala Ile Leu Phe Leu Pro Arg Lys Arg Ser Gln Ala Glu
60 65 70

cta tgt gca gac cca aag gag ctc tgg gtg cag cag ctg atg cag cat 351
Leu Cys Ala Asp Pro Lys Glu Leu Trp Val Gln Gln Leu Met Gln His
75 80 85

ctg gac aag aca cca tcc cca cag aaa cca gcc cag ggc tgc agg aag 399
Leu Asp Lys Thr Pro Ser Pro Gln Lys Pro Ala Gln Gly Cys Arg Lys
90 95 100 105

gac agg ggg gcc tcc aag act ggc aag aaa gga aag ggc tcc aaa ggc 447
Asp Arg Gly Ala Ser Lys Thr Gly Lys Lys Gly Lys Gly Ser Lys Gly
110 115 120

tgc aag agg act gag cgg tca cag acc cct aaa ggg cca tag 489
Cys Lys Arg Thr Glu Arg Ser Gln Thr Pro Lys Gly Pro
125 130

cccagtgagc agcctggagc cctggagacc ccaccagcct caccagcgct tgaagcctga 549

acccaagatg caagaaggag gctatgctca ggggccctgg agcagccacc ccatgctggc 609

cttgccacac tctttctcct gctttaacca ccccatctgc attcccagct ctaccctgca 669

tggctgagct gcccacagca ggccaggtcc agagagaccg aggagggaga gtctcccagg 729

gagcatgaga ggaggcagca ggactgtccc cttgaaggag aatcatcagg accctggacc 789

tgatacggct ccccagtaca ccccacctct tccttgtaaa tatgatttat acctaactga 849

ataaaaagct gttctgtctt cccacccaa 878

<210> 2
<211> 134
<212> PRT
<213> Homo sapiens

<400> 2

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Gly Ile Pro Arg Thr Gln Gly Ser Asp Gly Gly Ala Gln Asp Cys Cys
20 25 30

Leu Lys Tyr Ser Gln Arg Lys Ile Pro Ala Lys Val Val Arg Ser Tyr
35 40 45

Arg Lys Gln Glu Pro Ser Leu Gly Cys Ser Ile Pro Ala Ile Leu Phe
50 55 60

Leu Pro Arg Lys Arg Ser Gln Ala Glu Leu Cys Ala Asp Pro Lys Glu
65 70 75 80

Leu Trp Val Gln Gln Leu Met Gln His Leu Asp Lys Thr Pro Ser Pro
85 90 95

Gln Lys Pro Ala Gln Gly Cys Arg Lys Asp Arg Gly Ala Ser Lys Thr
100 105 110

Gly Lys Lys Gly Lys Gly Ser Lys Gly Cys Lys Arg Thr Glu Arg Ser
115 120 125

Gln Thr Pro Lys Gly Pro
130